



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/GB97/02751 <b>(22) International Filing Date:</b> 8 October 1997 (08.10.97)  <b>(30) Priority Data:</b> 9620940.8                      8 October 1996 (08.10.96)                      GB 9705911.7                      21 March 1997 (21.03.97)                      GB  <b>(71) Applicant (for all designated States except US):</b> INSTITUTE OF CHILD HEALTH [GB/GB]; 30 Guildford Street, London WC1N 1EH (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> FABRE, John, William [AU/GB]; Institute of Child Health, 30 Guildford Street, London WC1N 1EH (GB). GUSTAFSSON, Kenth, Tomas [SE/GB]; Institute of Child Health, 30 Guildford Street, London WC1N 1EH (GB). YUN, Sheng [CN/GB]; Institute of Child Health, 30 Guildford Street, London WC1N 1EH (GB).  <b>(74) Agents:</b> SILVESTON, Judith et al.; Abel & Imray, Northumberland House, 303-306 High Holborn, London WC1V 7LH (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> MUTANT CIITA MOLECULE AND USES THEREOF  <b>(57) Abstract</b> <p>A polypeptide that comprises the amino acid sequence of a class II trans activator (CIITA) protein from the N-terminus of which amino acid residues are missing such that the resulting polypeptide reduces the expression of MHC class II antigens is useful in the treatment of autoimmune disease and in the production of transgenic donor animals for xenografts and in the treatment of autoimmune diseases. Ribozymes targeting bases 1159-1161 of human CIITA are also useful, as are nucleic acids encoding the polypeptide and the ribozyme.</p>		

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## MUTANT CIITA MOLECULE AND USES THEREOF

Major Histocompatibility Complex (MHC)-encoded class II  
5 antigens are heterodimeric cell surface molecules, the function  
of which is to present peptides to CD4 positive thymocytes and  
peripheral T lymphocytes. They play a critical role in both  
thymic T cell ontogeny and the activation of peripheral immune  
responses (e.g. 1). Not surprisingly, genetic deficiency of  
10 MHC class II results in severe immunodeficiency, which has been  
called the "Bare Lymphocyte Syndrome" (e.g. 2,3).

Complementation studies with tissues from Bare Lymphocyte  
Syndrome patients have demonstrated a minimum of 4 recessive  
15 loci, all outside the MHC, and therefore presumably coding for  
trans-acting regulatory factors (4). One of these regulatory  
factors has been identified and designated the Class II Trans  
Activator (CIITA) (5). Since the original identification, the  
CIITA molecule has been shown to play a critical role in  
20 interferon gamma induced (6) and developmentally regulated (7)  
class II expression. In many situations, CIITA induction alone  
is sufficient to induce class II expression (8). Moreover,  
CIITA induces the expression of both the invariant chain and M  
locus class II molecules (9), both of which play crucial roles  
25 in the intracellular trafficking, peptide loading and cell  
surface expression of MHC class II molecules (10).

The promoter region of class II MHC genes in CIITA deficient  
patients is fully occupied by DNA binding proteins (11). This,  
30 together with the fact that CIITA shows no sequence homology to  
DNA binding proteins, suggests that it might bind to the  
proteins in the promoter region (5). The amino terminus of  
CIITA has an acidic region, with 30% glutamate or aspartate  
residues between amino acids 26-137. This is followed by three  
35 regions rich in proline, serine and threonine (amino acids 163-  
195, 209-237 and 261-322) (5). Transcription activators have  
been shown to have acidic, proline rich or glutamine rich  
regions (for review see 12), and it was therefore suggested  
that CIITA might consist of an acidic amino terminal activation

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domain and a carboxy terminal domain for binding to MHC class II region promoter proteins (5).

Model studies using fusion proteins of different regions of CIITA with the DNA binding region for a transcriptional activator have established that the acidic domain is indeed a potent transcriptional activator. All or most of the transcription activation activity could be ascribed to amino acids 1-125 (13) or 1-114 (14). The first two proline, serine and threonine rich regions by themselves (amino acids 166-232) had no transcriptional activation activity (13). Moreover, the carboxy terminal 813 amino acids, when fused to a general transcription activator, could specifically direct transcription from the DR $\alpha$  promoters, although less efficiently than the native CIITA molecule (14). However, the 831 carboxy terminal fragment was unable to suppress constituted class II expression.

Steimle et al. (5) and EP-0648364-A disclose the amino acid sequence of a human CIITA, the nucleic acid encoding it and a plasmid comprising the nucleic acid. The nucleic acid and amino acid sequences of human CIITA are set out in Figure 5 of the accompanying drawings and in SEQ.ID.NO.1 and SEQ.ID.NO.2, respectively.

It has been proposed in EP-0 648 364-A to use CIITA proteins and DNA sequences encoding those proteins to increase expression of MHC class II genes. It has also been proposed to use "antisense" DNA, RNA or ribozymes to repress MHC class II antigen expression.

The present invention is based on the observation that deletion of a region of a DNA sequence encoding an N-terminal portion of a CIITA molecule results in reduced expression of MHC class II antigens.



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The present invention provides a polypeptide that comprises the amino acid sequence of a class II trans activator (CIITA) protein from the N-terminus of which amino acid residues are absent such that the resulting polypeptide reduces the expression of MHC class II antigens. A polypeptide of the present invention may be referred to herein as a "deletion mutant CIITA polypeptide".

The present invention also provides a nucleic acid molecule that encodes a polypeptide of the present invention. A nucleic acid molecule of the present invention may be referred to herein as a "deletion mutant CIITA nucleic acid".

We have found that deletion of the first 151 amino acids from the N-terminus of human CIITA results strong suppression of MHC class II antigen synthesis both in cells that express the antigens constitutively and in cells that are susceptible to lymphokine induction of expression.

The deletion polypeptide was expressed from a mutated cDNA which incorporated the first six codons i.e. the start codon and the 5 codons corresponding to amino acids 2 to 6 of native human CIITA at the 5-end of the construct followed by a codon for isoleucine. At the junction of that oligonucleotide with the remainder of the construct, as a consequence of the manipulation, the native codon for a leucine residue at position 151 was replaced by a codon for isoleucine. The remainder of the construct i.e. from the codon for amino acid 152 to the end of the sequence is as shown in Figure 5. We consider the mutant polypeptide to have the first 151 amino acids deleted.

The amino acid sequence of the deletion mutant CIITA polypeptide and the nucleic acid sequence encoding it are set out in Figure 14 and in SEQ.ID.NOS.4 and 3, respectively.

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Hela cells, in which class II MHC antigen expression is induced by interferon gamma, were stably transfected with constructs comprising a cDNA encoding the 151 amino acid deletion mutant polypeptide in the pcDNA3 vector or were transiently transfected with constructs comprising the cDNA encoding the 151 amino acid deletion mutant CIITA polypeptide in the pCEP4 vector. (The 151 deletion mutant polypeptide is described in detail in Example.) The transfected cells showed a 50-90% suppression of MHC class II antigen induction by interferon gamma as assessed by flow cytometry and a suppression of HLA-DR mRNA expression by RT-PCR. Transient transfection studies using constructs comprising the cDNA encoding the 151 amino acid deletion mutant CIITA polypeptide with the B-cell line DoHH2, which expresses MHC class II antigens constitutively at high levels, resulted in an up to 89% reduction of cell surface expression of MHC class II antigens over 5 days, and an almost complete suppression of HLA-DR mRNA synthesis. Transfection with an empty expression vector or with a modified version of the deletion construct having no initiation codon revealed no reduction of MHC class II antigen expression in either cell type.

Without being limited to the following theory, we believe that the removal of an N-terminal region of a CIITA molecule, in particular the acidic activator domain, results in a deletion mutant CIITA polypeptide that has lost its transcription activation activity and but that is still able to bind to the regulatory proteins in the promoter region of the MHC class II antigen gene and hence to act as an inhibitor by competing with endogenously produced CIITA for binding to proteins in the promoter region. It may also be relevant that transcription activators are frequently active as dimers or higher multimers (e.g. 16, 25, 26). If this is the case with CIITA, it is possible that in addition to competing for binding to proteins in the promoter region, the deletion mutant CIITA polypeptide

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may be either unable to form multimers, or dimers or multimers incorporating a mutant CIITA are functionally compromised. In such circumstances, the suppressive effect of mutated CIITA molecules would be far greater than would be expected from the relative concentrations of the endogenous and mutated forms.

As indicated above, we have found that deletion of the first 151 amino acids from the N-terminus of a human CIITA molecule results in a polypeptide that reduces the expression of MHC class II antigens. In that polypeptide, the acidic activator domain is deleted but the proline/serine/threonine rich domains are retained.

To produce a deletion mutant CIITA polypeptide of the present invention, it appears to be preferable to delete all or most of the acidic activator domain from the N-terminus of a CIITA protein. As indicated above, all or most of the transcription activation activity has been ascribed to amino acids 1-125 (13) or 1-114 (14). It is therefore preferable to delete the amino acids in that region, for example, it may be advantageous to delete at least amino acids 1-114, for example, at least amino acids up to and including amino acid 125. If too little of the acidic activator domain is deleted, transcription activation activity may be retained.

As indicated by our own results, amino acids downstream of the transcription activator domain may also be deleted. However, deletion of too many downstream amino acids may result in loss of inhibitory activity. It appears to be advisable to retain most and preferably all of the proline/serine/threonine rich domains, as a CIITA polypeptide lacking those domains was unable to suppress constituted MHC class II antigen expression (14). The proline/serine/threonine rich domains may play a critical role in the conformation of the remainder of the protein and/or they may themselves be directly involved in critical protein/protein interactions. It is therefore

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preferable that a deletion does not extend into the proline/-  
serine/threonine rich domain. The first proline/serine/-  
threonine rich domain appears to start at around amino acid 163  
to 166. It is preferable, therefore, that a deletion does not  
5 extend to the first amino acid of the first proline/serine/-  
threonine rich domain, for example, does not extend beyond  
amino acid 165, for example not beyond amino acid 162. It may  
be preferable that a deletion does not extend too close to the  
proline/serine/threonine rich domains, for example it may be  
10 preferable that a deletion does not extend beyond about an  
amino acid 164, for example, not beyond amino acid 160 for  
example, not beyond amino acid 156.

We have demonstrated that the deletion of 151 amino acids from  
15 the N-terminus results in a deletion mutant CIITA polypeptide  
having the ability to reduce expression of MHC class II  
antigens. As indicated above, more than 151 amino acids or  
fewer than 151 amino acids may be deleted from the N-terminus,  
provided the desired activity is retained, that is to say,  
20 expression of MHC class II antigens is reduced.  
Recommendations for the largest and smallest size of deletions  
are set out above. By way of example, the N-terminus the  
deleted polypeptide may start at amino acid 114 or at any amino  
acid downstream from amino acid 114 for example, amino acid 125  
25 or downstream thereof, for example, amino acid 135 or  
downstream thereof, for example, amino acid 140 or downstream  
thereof. Preferably the N-terminus should not extend beyond  
amino acid 164, for example not beyond amino acid 160, for  
example not beyond amino acid 156. An N-terminus in the region  
30 of from amino acid 140 to 152 may be particularly useful.

In the case of a non-human CIITA, for example, a porcine CIITA,  
an equivalent number of amino acids may be deleted, or a  
different number may be deleted, provided a reduction in the  
35 expression of MHC class II antigens is achieved. As for a  
human CIITA protein, the deletion should preferably encompass

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the acidic activator region but not extend far into, and preferably should not extend into the proline/serine/threonine rich domains.

5 Any candidate deletion mutant CIITA polypeptide or deletion mutant CIITA nucleic acid encoding such a polypeptide may be readily tested for its ability to reduce expression of MHC class II antigen activity, either constitutive expression or induced expression, by any of the methods known in the art, for  
10 example, by any of the methods described herein. General examples using inducible expression in Hela cells and constitutive expression in B cells are given above. Detailed protocols are given in the Example below.

15 A CIITA protein may be of any animal origin, for example, a human, porcine, bovine, rodent or baboon CIITA, or may be a variant thereof in which there are substitutions, deletions or additions to the amino acid sequence that do not substantially affect the transcription activation activity of the protein.

20 As a deletion mutant CIITA polypeptide of the invention comprises part of the amino acid sequence of a CIITA protein, the amino acid sequence of the deletion mutant CIITA polypeptide will reflect any such substitutions, deletions and additions.

25 A nucleic acid molecule of the present invention is any molecule that encodes a deletion mutant CIITA polypeptide of the present invention. It may be DNA, for example, genomic or preferably cDNA, or may be RNA, for example, mRNA or may have  
30 the same nucleic acid sequence as genomic DNA, cDNA or mRNA. Such a molecule may be derived, directly or indirectly, from a naturally occurring sequence that encodes a CIITA or from a degenerate version of such a sequence. It may be derived, directly or indirectly, from an allele of naturally-occurring  
35 CIITA sequence or from a sequence that hybridises to a sequence that encodes a CIITA. It may be obtained from natural sources

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or it may be recombinant or synthetic.

The cDNA and predicted amino acid sequence of a human CIITA protein are given in Figure 5 of the accompanying drawings, and in SEQ.ID.NOS.1 and 2, respectively.

Nucleic acid sequences encoding CIITA polypeptides may be obtained from other species by standard methods, for example, as described in Sambrook J, Fritsch EF and Maniatis T, Molecular Cloning: A laboratory manual, Cold Spring Harbor 1989 using primers and/or probes derived from the human sequence. Examples of human-derived PCR primers capable of detecting porcine CIITA cDNA are given in Example 3 herein. The invention accordingly provides nucleic acid that hybridises selectively to a nucleic acid of figure 14 (SEQ.ID.NO.3)

The present invention also relates to ribozymes directed at CIITA mRNA.

Ribozymes are RNA enzymes that cleave RNA at preferred triplet sequences, for example, NUX for hammerhead ribozymes. The specificity of a ribozyme for any particular RNA is dictated by the sequence of the ribozyme's antisense regions (usually 7-10 bases at the 3' and the 5' ends), which enable the ribozyme to bind to complementary regions of the target RNA and then to cleave it. Cleavage can disrupt mRNA function, but more importantly it results in rapid mRNA degradation, and thereby diminishes the expression of the protein product. There are various types of ribozymes, including the so-called hammerhead ribozyme. Ribozymes and methods for their production are well known see, for example, Tanner et al (24), Ellis et al (31) and Larsson et al (32).

Once an mRNA sequence is known, the potential exists for targeting it with ribozymes. However, few target sites are effective. The reasons for this are not clear, but the

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secondary structure of the mRNA might be responsible in part, by making the target sequences inaccessible to the ribozymes. Computer predictions of mRNA secondary structures are often used in an attempt to identify open loops, which might be more effectively targeted, but this is a very imprecise field. Finding an effective ribozyme is largely a matter of trial and error, and only occasionally are ribozymes effective.

The present invention provides a ribozyme directed at bases 1159-1161 (GUA) of human CIITA mRNA.

The ribozyme is, for example, a hammerhead ribozyme. The antisense arms of the ribozyme, are TGTTGGA at the 5' end and ACGTGTC at the 3' end.

The present invention also provides a ribozyme targeted at a non-human CIITA mRNA. The target base sequence may be at the position in that mRNA equivalent to bases 1159-1161 (GUA) of human CIITA mRNA or at another sequence.

The human ribozyme of the invention markedly suppresses induced MHC class II expression in transfected human cells, in contrast to ribozymes directed against other human CIITA sequences.

The expression of polymorphic donor MHC class II antigen molecules is of fundamental importance in graft rejection processes and also in autoimmune diseases. Some cells, for example, interstitial dendritic cells, express MHC class II antigens constitutively, whereas others are susceptible to MHC class II antigen induction following lymphokine stimulation.

The acute shortage of human organs for transplantation has resulted in intensive efforts to identify additional sources of donor organs. The possibility of using other species, in particular the pig, as a novel source of grafts for man has been perceived in recent years as the only avenue offering

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substantive hope of alleviating the donor shortage. Intensive research efforts have brought the field to a stage where clinical trials of porcine organ xenografts to man might soon begin<sup>22</sup>.

5

The major immediate problem with porcine organ xenografts to man and to other old world primates has been natural antibody- and complement-mediated hyperacute rejection, which destroys organ xenografts within a few minutes or  
10 hours of transplantation. This potent and seemingly insuperable barrier appears now to have been overcome by the use of transgenic pig donors expressing human complement regulators<sup>23</sup>.

15 Now that one can look beyond hyperacute rejection, it is clear that there are important barriers still to be overcome. For example, the longer term survival of transgenic porcine organ xenografts in old world monkeys requires high levels of immunosuppression<sup>33</sup>. Some of these barriers are likely to  
20 encompass rejection mechanisms not seen with allografts, in particular involving NK cells, macrophages and granulocytes<sup>24-26</sup>.

It has been suggested that class II MHC expression on vascular  
25 endothelial cells plays a critical role in the long-term immunogenicity of transplanted human organs<sup>28</sup>. Donor Major Histocompatibility Complex (MHC) Class II molecules may play a crucial role in allotransplantation as activators of  
30 recipient CD4+ T cells via the direct pathway, i.e., T cell recognition of intact donor MHC class II molecules on donor antigen presenting cells (APC). Donor MHC class II  
molecules may also contribute to the pool of allogeneic donor peptides for activation of recipient CD4+ T cells via  
the indirect pathway, i.e., T cell recognition of donor  
35 peptides presented by recipient APC (for review see ref.<sup>1</sup>). In the context of xenotransplantation, the contribution of



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donor MHC class II molecules to the indirect pathway is presumably relatively insignificant, given the vast pool of other foreign proteins present in the donor organ. However, in some donor-recipient species combinations, xenogeneic MHC class II molecules might play a crucial role in xenograft rejection by activation of the recipient's direct T cell recognition pathway.

Direct T cell recognition is weak or absent in some species combinations, presumably as a consequence of physiological incompatibilities for vital co-receptor, costimulator, adhesion or cytokine interactions (e.g. reference <sup>2</sup>). It had been hoped that such incompatibilities would reduce or abolish the strength of direct T cell recognition responses in clinical xenotransplantation. However, the accessory interactions for direct T cell recognition by human T cells of porcine APCs are largely intact<sup>3</sup>.

We consider that one of the important barriers to the use of xenografts will be that of T cell-mediated rejection, involving both direct T cell recognition of SLA-DR and SLA-DQ antigens, and indirect T cell recognition of the many thousands of foreign proteins present in porcine organs<sup>1,27</sup>.

In transplantation, two donor cell types are likely to be the major stimulants of direct T cell recognition: the migratory leucocytes known as interstitial dendritic cells and MHC class II positive VEC <sup>29,30</sup>. Whereas donor interstitial dendritic cells are transient components of graft, emigrating within 1 or 2 weeks of transplantation<sup>31</sup>, VECs are present for the life time of the graft.

It has been proposed that the MHC class II positive VECs of allografted organs represent a strong, constant stimulus for direct T cell allorecognition in clinical transplantation, and that the suppression of MHC class II expression on VECs

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in allografted organs might markedly reduce the strength of rejection response, in particular chronic rejection responses<sup>32</sup>.

5 It is of particular interest that direct recognition by human T cells of MHC class II positive porcine vascular endothelial cells (VECs) is unusually potent, with T cell precursor frequencies being greater for the direct xenogeneic than the direct allogeneic response with VEC  
10 stimulators<sup>4</sup>. Direct T cell xenorecognition of donor VEC therefore is likely to be of particular importance in the transplantation of porcine organ xenografts to man. This species difference is almost certainly the basis for the higher precursor frequency of human CD4+ T cells for direct  
15 xenorecognition of porcine VECs as compared to direct allorecognition of human VECs<sup>4</sup>.

Because VECs are permanent components of grafts, and because both the pig<sup>6</sup> and human<sup>7</sup> express MHC class II antigens on  
20 their VECs, this species difference in CD86 expression is likely to result in greater long-term immunogenicity of porcine xenografts as compared to allografts in the clinical situation. We therefore consider that class II molecules therefore potentially represent important targets for immune  
25 regulation.

Both deletion mutant CIITA polypeptides and ribozymes of the present invention strongly suppress the expression of polymorphic MHC class II antigen molecules. Accordingly, the  
30 expression of a deletion mutant CIITA polypeptide or of a ribozyme of the invention in the vascular endothelium or other cells of transplanted tissues and organs will markedly suppress rejection responses, whether in human-to-human allografts or in xenografts, for example, pig-to-human xenografts.

35

The present invention accordingly enables the production of

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transgenic animals having reduced expression of MHC class II antigens. Such animals may be used as a source of organs or tissue for xenogenic transplantation and/or of cells, for example, for universal cell transplants. Pigs are particularly useful as donor animals for xenografts but other animals are also used as donors for cells, tissues and organs, for example, foetal calf cells and baboon bone marrow cells are useful in transplantation. Other animals, for example, rodents, may be also be used as donor animals.

The present invention accordingly provides a transgenic animal, for example, a transgenic pig, at least some of the cells of which comprise a stably incorporated, functional DNA sequence that encodes a polypeptide that comprises the amino acid sequence of a class II trans activator (CIITA) protein from the N-terminus of which amino acid residues are absent such that the resulting polypeptide reduces the expression of MHC class I antigens, or that encodes a ribozyme of the present invention.

The invention also includes progeny of such a transgenic animal.

The present invention further provides a method for producing a transgenic animal of the invention, which comprises stably incorporating a functional DNA sequence that encodes a polypeptide that comprises the amino acid sequence of a class II trans activator (CIITA) protein from the N-terminus of which amino acid residues are missing such that the resulting polypeptide reduces the expression of MHC class II antigens, or that encodes a ribozyme of the present invention and, optionally, breeding the transgenic animal to produce progeny thereof.

For maximum efficiency in a transgenic animal, the nucleic acid should generally encode a CIITA polypeptide of the same species as the host animal. For example, in the case of a transgenic pig, the nucleic acid sequence should generally encode a

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porcine CIITA polypeptide. However, we have found, surprisingly, that the deletion mutant CIITA polypeptide appears to be effective across species, for example, human deletion mutant CIITA is effective in the pig. Accordingly, in a transgenic animal, a nucleic acid may encode a deletion mutant CIITA polypeptide of the same species or of a different species.

A ribozyme of the present invention is unlikely to be effective in a different species because of species differences in the target site. The target base sequence in the mRNA of a different species may be at the position in that mRNA equivalent to bases 1159-1161 (GUA) of human CIITA mRNA, or it may be at a different site.

MHC class II antigen expression may be suppressed globally according to the present invention, but the resulting immune deficiency in a potential donor animal is generally undesirable. It is therefore advantageous to incorporate the nucleic acid sequence under the control of a tissue-specific promoter in order to down-regulate MHC class II antigen production in a specific tissue only. It is particularly advantageous to suppress MHC-II expression on the vascular endothelial cells of a xenograft. Accordingly, a vascular endothelial cell-specific promoter is preferably used in a transgenic animal. Alternatively, there may be used a promoter specific for the organ to be transplanted, for example, a cardiac-specific promoter may be used for a heart or heart valve xenograft.

Tissue-specific and organ-specific promoters are well known. An example of a vascular endothelial cell-specific promoter is the ICAM-2 promoter, see Kowan et al (33) for the human promoter and Xu et al (34) for the mouse ICAM-2 promoter. The ICAM-2 promoter region from one species, for example, the human or mouse ICAM-2 promoter region may be used as such or may be

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modified for use in another species, for example, a pig. An example of a modified mouse ICAM-2 promoter suitable for use in a pig may be prepared by PCR using, the mouse sequence -676 to -645 as the 5' primer and the mouse sequence -44 to -74 as the 3' primer.

The present invention also provides a cell, tissue or organ that comprises a stably incorporated DNA molecule that encodes a polypeptide that comprises the amino acid sequence of a class II trans activator (CIITA) protein from the N-terminus of which amino acid residues are missing such that the resulting polypeptide reduces the expression of MHC class II antigens, or that encodes a ribozyme of the present invention.

Such a transgenic cell, tissue or organ may have been obtained from a transgenic animal of the present invention or may have been produced directly by the stable introduction of a nucleic acid molecule of the present invention that is to say, a nucleic acid molecule that encodes a polypeptide that comprises the amino acid sequence of a class II trans activator (CIITA) protein from the N-terminus of which amino acid residues are missing such that the resulting polypeptide reduces the expression of MHC class II antigens, or that encodes a ribozyme of the present invention.

A transgenic cell of the present invention is especially in the form of a cell line that can be maintained in vitro. A cell or cell line of the invention is especially a cell or cell line suitable for use in transplantation. Analogously, a transgenic tissue or organ, for example skin, may be in a form that can be grown or maintained in vitro.

As indicated above, transgenic pigs that express human complement receptors have been produced (23). It is particularly advantageous to produce double transgenic pigs that express both human complement receptors and a deletion

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mutant CIITA polypeptide of the present invention.

Accordingly, the present invention provides a transgenic animal, for example, a transgenic pig, at least some of the cells of which comprise a stably incorporated, functional DNA sequence that encodes a polypeptide that comprises the amino acid sequence of a class II trans activator (CIITA) protein from the N-terminus of which amino acid residues are absent such that the resulting polypeptide reduces the expression of MHC class II antigens, or that encodes a ribozyme of the present invention, and at least some of the cells of which, for example, the same cells, comprise a stably incorporated functional DNA sequence that encodes a human complement receptor.

Double transgenic pigs, involving a human regulator of complement (to prevent hyperacute rejection) and a mutated human CIITA construct of the present invention preferably under the control of a constitutive VEC specific promoter (to suppress direct T cell xenorecognition), will provide an important advance in the development of porcine xenografts for clinical transplantation.

The present invention also comprises such double transgenic cells, organs and tissues, and methods for the production of such cells, organs, tissues and animals.

The DNA sequence encoding a human complement receptor may be introduced independently of the DNA sequence encoding a deletion mutant CIITA polypeptide of the present invention, or both may be introduced together. The method described<sup>23</sup> or in reference<sup>37</sup> may be used for introduction of DNA sequence of present invention and/or for the introduction of the DNA sequence encoding the human complement receptor. Optionally a DNA construct or vector may comprise both DNA sequences, generally arranged such that each polypeptide is expressed

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separately rather than as a fusion protein.

As described above, a polypeptide or ribozyme of the present invention may be expressed in vivo in an animal.

5 Alternatively, a polypeptide or ribozyme of the present invention may be expressed by a prokaryotic or eukaryotic host cell in vitro. The polypeptide may then be isolated and purified for use, for example, in a pharmaceutical composition.

10 The present invention provides a pharmaceutical composition that comprises a deletion mutant CIITA polypeptide of the present invention or a nucleic acid encoding a deletion mutant CIITA polypeptide of the present invention, in admixture or  
15 conjunction with a pharmaceutically suitable carrier. The invention also provides a pharmaceutical composition that comprises a ribozyme of the present invention or a nucleic acid encoding a ribozyme of the present invention, in admixture or  
conjunction with a pharmaceutically suitable carrier.

20 Methods for producing suitable constructs and vectors for in vivo and in vitro expression are well known, as are methods for introducing such constructs and vectors into host cells. The present invention provides such constructs and vectors.

25 The present invention accordingly provides a construct that comprises a nucleic acid molecule of the present invention suitable for incorporation in a vector or suitable for direct insertion into a host cell.

30 The present invention also provides a vector, especially an expression vector, that comprises a nucleic acid of the present invention operably linked to appropriate control sequence(s). The vector may be suitable for incorporation into a prokaryotic or eucaryotic host for expression of the polypeptide or  
35 ribozyme in vitro. Alternatively, the vector may be suitable for incorporation into a host cell, tissue, organ or animal for

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expression of the polypeptide or ribozyme in vivo. A tissue-specific promoter, especially a vascular endothelial cell-specific promoter may be present.

5 Vectors, host cells and methods for the production of constructs, vectors and transformed host cells are well known in the art, see for example, Sambrook J. Fritsch EF and Maniatis T, Molecular Cloning: A laboratory manual, Cold Spring Harbor 1989. Methods for introducing DNA into animals to  
10 produce functional transgenic animals, including DNA constructs and vectors for the stable incorporation of DNA sequences into cells for use in the production of transgenic animals are also well known, see for example, Hogan B, Constantini F, Lacy E, Manipulating the mouse embryo: A laboratory manual, Cold Spring  
15 Harbor 1986. methods for producing transgenic pigs are described in McCurry et al.<sup>23</sup> and in Logan & Martin<sup>37</sup>. The standard pronuclear microinjection technique, described for mice by Hogan et al. may be used successfully in pigs <sup>23,37</sup>.

20 It may be advantageous to incorporate further elements, for example, the nuclear localisation signal (NLS) of the large T antigen of SV40, in a construct or vector<sup>14</sup>. It may also be advantageous to adopt measures that improve or increase the stability of mRNA in mammalian cells.

25 The present invention provides the use of a nucleic acid molecule of the present invention in the production of a transgenic cell, tissue, organ or animal.

30 The present invention further provides a nucleic acid of the present invention for use in the manufacture of a construct or vector for use in the production of a transgenic cell, tissue, organ or animal.

35 As indicated above, the nucleic acid may encode a CIITA polypeptide from the same species to which it will be



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introduced, or it may be from a different species, for example, a construct for use in the production of a transgenic pig may comprise nucleic acid encoding a human deletion mutant CIITA polypeptide or encoding a porcine deletion mutant CIITA polypeptide.

The suppression of SLA-DR and SLA-DQ expression on the VECs of porcine organ xenografts with the mutant human CIITA constructs of the present invention enable the reduction and even complete abolition of the direct T cell recognition response in human recipients of porcine xenografts, thus facilitating the long-term acceptance of porcine xenografts with clinically acceptable levels of immunosuppression.

It is important to note that total suppression of MHC class II expression on APCs is not required for substantial reduction in their antigen-presenting capacity. For example, Hatano et al<sup>34</sup> were able to abolish the capacity of B cells to present antigen to T cell clones by suppressing MHC class II expression by only ~80%, using synthetic antisense oligonucleotides to MHC class II mRNA. In our work, the residual low levels (~5%) of SLA-DR and SLA-DQ in porcine PIEC were unable to stimulate direct xenorecognition by human T cells.

CIITA has recently been shown to play a role in upregulating the expression of HLA class I heavy chain genes, but not the other genes involved in the cell surface expression of MHC class I molecules (eg.  $\beta_2$  microglobulin, TAP, LMP)<sup>35,36</sup>.

However, in our studies, suppression of CIITA did not influence porcine MHC class I expression. In addition, although CIITA independent expression of HLA-DQ has been demonstrated in EBV transformed B cells<sup>13</sup>, we did not see a differential effect on SLA-DR or SLA-DQ in our studies.

MHC class II antigens are also implicated in autoimmune

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disease. The down-regulation of MHC class II antigen expression is therefore useful in the treatment of autoimmune disease as well as in the production of transgenic material for xenografts.

5

Accordingly, the present invention provides a method of reducing the expression of MHC class II antigens in a human or non-human animal, which comprises administering to the human or other animal an effective amount of a polypeptide or ribozyme of the present invention.

10

The present invention also provides a pharmaceutical composition which comprises a deletion mutant CIITA polypeptide or ribozyme of the present invention of admixture or conjunction with a suitable carrier.

15

The human or other animal to be treated may have an autoimmune disease. Alternatively, a non-human animal to be treated may be intended for use as a xenograft donor.

20

A nucleic acid molecule of the present invention, encoding either a mutant CIITA polypeptide or a ribozyme of the invention may be used in gene therapy for reducing the expression of MHC class II antigens, for example, in the treatment of an autoimmune disease in a human or in a non-human animal. Administration of an appropriate amount of the nucleic acid to a subject will result in reduction of the expression of MHC class II antibodies and hence a reduction in the undesirable immune response.

25

30

The nucleic acid may be administered in a targeted manner such that local immune response suppression is achieved. For example, in the case of arthritis, it may be useful to suppress the immune response in a selected joint, for example, a knee wrist or neck joint. The use of appropriate tissue-specific promoters may also be advantageous, for example, it may be

35

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appropriate to use an insulin-specific promoter in the treatment of diabetes.

5     Suitable methods for administering nucleic acids for gene therapy, including targeted gene therapy, are known, as are suitable constructs and vectors. Such a method of gene therapy is part of the present invention.

10    The present invention also provides a pharmaceutical composition which comprises a nucleic acid molecule of the present invention in a suitable form for use in gene therapy.

15    The present invention further provides the use of a nucleic acid molecule of the present invention in the reduction of the expression of MHC class II antigens, for example, in the treatment of an autoimmune disease.

20    The present invention also provides a nucleic acid molecule of the present invention for use in the manufacture of a medicament for use in gene therapy, for example, in the treatment of an autoimmune disease, or for use on the production of a transgenic animal.

25    The present invention further provides the use of a deletion mutant CIITA polypeptide or a ribozyme of the present invention in the reduction of the expression of MHC class II antigens, for example, in the treatment of an autoimmune disease.

30    The present invention further provides an antibody to a polypeptide of the present invention, either polyclonal or monoclonal.

35    The present invention further provides a method of animal-to-human transplantation, wherein the transplanted material, for example, cells, tissue or organ, is derived from a transgenic animal of the present invention.

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As indicated above in the context of transgenic animals, a deletion mutant CIITA polypeptide of the present invention appears to function across species. Accordingly, for any of the embodiments of the present invention the deletion mutant CIITA polypeptide as such or as encoded by a nucleic acid may be of the same species as the intended recipient or may be of another species.

The following is a brief description of the drawings:

**Fig. 1 Mutated CIITA constructs**

A. The pcDNA3mutCIITA2 construct was made by inserting a subclone from the original full-length cDNA, between a NotI site at position 1340 and a XhoI site at position 4543, followed by the insertion of a PCR product incorporating an introduced EcoRV site at position 567 and the NotI site at position 1340. This is a control construct, without an initiation codon, and would not yield CIITA protein.

B. The pcDNA3mutCIITA3 and pcDNA3mutCIITA4 constructs were made by inserting synthetic double stranded oligonucleotides between the EcoRI and EcoRV sites. ATG means initiation codon, and NLS means Nuclear Localisation signal.

C. The pCEP4mutCIITA2 was made by subcloning the fragment from EcoRI to XhoI from pcDNA3mutCIITA2 into pCEP4. Similarly, pCEP4mutCIITA3 and pCEP4mutCIITA4 were made by transferring the EcoRI/XhoI fragment from pcDNA3mutCIITA3 and pcDNA3mutCIITA4 respectively.

**Fig. 2 Base-line studies on Hela and DoHH2 cells**

The Hela and DoHH2 cell lines were studied by RT-PCR (A) and flow cytometry (B) before and 24 hours after interferon gamma treatment. The cells used for the RT-PCR studies in A were from the same cultures used for flow cytometry studies in B.

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A: Hela cells before (lane 1) and after (lane 2) interferon gamma treatment and DoHH2 cells before (lane 3) and after (lane 3) interferon gamma treatment (lane 4). B: Flow cytometry profiles with the irrelevant antibody (...) are given for Hela cells in panel 2 and for DoHH2 cells in panel 3. Hela cells were examined for class II MHC antigens (panel 1) and class I MHC antigens (panel 2), both before interferon gamma treatment (open profiles) and after interferon gamma treatment (shaded profiles). DoHH2 cells (panel 3) were studied for class II (...) and class I (----) MHC antigens, as indicated, which was not significantly altered by interferon gamma treatment (not shown).

**Fig. 3 Suppression of class II induction in Hela cells**

Hela cell clones stably transfected with the pcDNA3mutCIITA2 (clone 1), pcDNA3mutCIITA3 (clone 2) and pcDNA3mutCIITA4 (clone 3), and Hela cell cultures transiently transfected with pCEP4CIITA2 (culture 1) and pCEP4mutCIITA4 (culture 2) were studied by RT-PCR (A) and flow cytometry (B). The cells used for the RT-PCR studies in A were from the same cultures used for flow cytometry studies in B. A. RT-PCR studies on Hela cells 24 hours after treatment with interferon gamma. B. Cells were analysed for class II antigens (left column) and class I antigens (right column), with the control antibody (...) shown in the right column. Open profiles correspond to cultures before interferon gamma treatment, shaded profiles after interferon gamma treatment.

**Fig. 4 Suppression of constitutive class II expression in DoHH2 cells**

The DoHH2 B lymphoblastoid cell line was transiently transfected with empty pcDNA3 vector (1), pcDNA3mutCIITA2 containing a non-functional CIITA gene (2), pcDNA3mutCIITA3 (3) and pcDNA3mutCIITA4 (4.) RT-PCR (A) and flow cytometry studies (B) were performed 5 days following transfection. The cells used for the RT-PCR studies were from the same cultures used

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for flow cytometry. In B, the cultures as indicated were stained for class II MHC antigens. The profile for the control antibody (...) is given for cells transfected with the empty pcDNA3 vector shown. Profiles for class I expression were the same for all cultures (not shown).

**Fig. 5 Nucleotide and predicted amino acid sequence of CTIIA DNA**

The complete nucleotide sequence of the cDNA clone pDVP10-1 and the deduced amino acid sequence of CIITA are shown. The 5'-ends of three independent clones are indicated by "#", the upstream in-frame stop codon and the stop-codon at nt position 3506 are indicated by "\*". The N-terminal regions rich in glutamate/aspartate (marked "acidic") and the stretches rich in proline/serine/threonine (marked "I, II,III") are overlined. The ATP/GTP binding cassette is double underlined. The sequences and their description are taken from Steimle V, Otten LA, Zufferey M and Mach B, Cell, 75, 135-146, 1993. The sequences are also shown in EP-0 648 836-A. The nucleic acid sequence forms SEQ.ID.NO.1 and the amino acid sequence forms SEQ.ID.NO.2.

**Figure 6 Suppression of induced class II expression in ECV-304 cells by a ribozyme**

A. Class II antigen synthesis was induced in ECV-304 by human interferon gamma using 500 and 1,000 unites. Flow cytometry was carried out 24,48 and 72 hours after the interferon treatment. In the legend to the Figure 24, 48 and 72 denotes the number of hours. 500U and 1000U respectively denote 500 and 1000 units of human interferon gamma.

B. ECV-304 cells were transfected with the pcDNA3 VECTOR CONTAINING THE GUA ribozyme under the control of the CMV promoter. The transfected cells were treated with interferon and flow cytometry was carried out as in A.

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**Figure 7    Suppression of induced class II expression in  
                 ECV-304 cells by a ribozyme**

A.    MHC class II expression is shown 72 hours after stimulating non-transfected ECV-40 cells (ECV stimulated with gIFN) and ribozyme-transfected ECV-40 cells (ECV+rnz+gIFN) with 1000 units of human interferon gamma. Expression in untreated cells is also shown (ECV untreated).

**Figure 8.    Human CIITA constructs**

The full length construct in pcDNA3 is illustrated in (a). The control mutCIITA2 construct, lacking an initiation codon, is illustrated in (b). The mutCIITA3 and mutCIITA4 constructs, containing synthetic oligonucleotides to support translation of the mRNA (by the ATG initiation codon) and translocation of the protein to the nucleus (by the nuclear localisation signal of the large T antigen of SV40) are illustrated in (c).

**Figure 9.    Human CIITA is effective in a porcine endothelial cell line**

The PIEC cell line was transfected with the full length human CIITA construct and studied by flow cytometry (A) and RT-PCR (B). The cultures used for the RT-PCR studies in (B) were the same cultures used for the flow cytometry studies in A.

A: normal PIEC (upper panel); PIEC transiently transfected with full length human CIITA (middle panel); a representative PIEC clone stably transfected with full length human CIITA (lower panel). The transiently transfected culture was placed in selective medium at day 2, and examined at day 7. The antibodies used were: control IgG<sub>1</sub> (⌘), SLA-DR (grey), SLA-DQ

( ----- ), SLA class I ( . . . . ). B: lanes 1, 2 and 3 represent normal PIEC, transiently transfected PIEC and a stably transfected PIEC clone respectively. 50ng of mRNA from each culture was used for the RT-PCR studies.

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**Figure 10. Base line studies on PIEC and L23 cells**

The porcine endothelial cell line (PIEC) and the porcine B cell line L23 were studied by flow cytometry (A) and RT-PCR (B). The cultures used for the flow cytometry studies in A were the same as those used for the RT-PCR studies in B. A: PIEC cells before (upper panel) and 72 hours after (middle panel) treatment with recombinant porcine interferon gamma. Normal L23 cells are shown in the lower panel. The antibodies used were: control IgG<sub>1</sub> (...), SLA-DR (grey), SLA-DQ (----), SLA class I (.....). B: lanes 1, 2 and 3 represent the normal PIEC, the interferon gamma treated PIEC, and normal L23 cells respectively. 50ng of mRNA from each culture was used for the RT-PCR studies.

**Figure 11. Suppression of interferon gamma induced MHC class II expression**

PIEC clones stably transfected with empty vector (clone 1), control pcDNA3mutCIITA2 (clone 2), pcDNA3mutCIITA3 (clone 3) and pcDNA3mutCIITA4 (clone 4) were studied by flow cytometry (A) and semi-quantitative RT-PCR (B). The cells used for the RT-PCR and flow cytometry studies came from the same cultures. All cultures had been exposed to recombinant porcine interferon gamma for 72 hours. A: The cells were analysed for SLA-DR (left hand column), SLA-DQ (middle column) and SLA class I (right hand column). The antibodies used were: control IgG<sub>1</sub> (....), SLA-DR (grey), SLA-DQ (---), SLA class I (.....). B: The amount of mRNA used for RT-PCR is shown above the wells. Lane 1 corresponds to clone 1, and so on for the other lanes.

**Figure 12. Suppression of constitutive MHC class II expression**

The L23 cell line was transiently transfected with empty pcDNA3 vector (culture 1), pcDNA3mutCIITA2 (culture 2), pcDNA3mutCIITA3 (culture 3) and pcDNA3mutCIITA4 (culture 4). Selection was applied at day 2 and the culture studied 7



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days later by flow cytometry (A) and semi-quantitative RT-PCR (B). The cells used in the flow cytometry and RT-PCR studies came from the same cultures. A: The cultures were examined for SLA-DR (left hand column), SLA-DQ (middle column) and SLA class I (right hand column). The antibodies used were: control IgG<sub>1</sub> (....), SLA-DR (grey), SLA-DQ (----), SLA class I (••••). B: The amount of mRNA used for RT-PCR is shown above the wells. Lane 1 corresponds to culture 1, and so on for the other lanes.

**Figure 13. Suppression of human anti pig direct T cell xenorecognition**

Human CD4<sup>+</sup> T cells were incubated for 4-6 days with normal porcine PIEC cells or PIEC cells which had previously been treated for 3 days with rpoIFN<sub>γ</sub>, as indicated. To check APC depletion, unfractionated PBL and CD4<sup>+</sup> T cells were incubated with 5 μg/ml of PHA and harvested 3 days later. <sup>3</sup>H thymidine was added for the last 18 hours of culture. The values represent means ± SEM of triplicate values.

**Figure 14. 151 Deletion mutant**

The nucleic acid sequence encoding the 151 deletion mutant described in Example 1 and the deduced amino acid sequence are set out.

The nucleic acid sequence forms SEQ.ID.NO.3 and the amino acid sequence forms SEQ.ID.NO.4.

The following non-limiting Example illustrates the invention.

**EXAMPLE 1**

**Cell culture**

The HeLa cell line was maintained in DMEM and the DoHH2 human B lymphoma cell line in RPMI 1640 (both media from Gibco BRL, Paisley, UK). In each case the medium was supplemented with 10% foetal calf serum (FCS), 2mM glutamine, 100 units/ml penicillin and 100μg/ml streptomycin. For passaging and all other manipulations, the HeLa cells

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were recovered by scraping. All cultures were at 37<sup>0</sup>C in 95% air/5% CO<sub>2</sub>.

#### Induction of MHC class II antigens

5 Human recombinant interferon gamma (a kind gift from Dr. N Klein, Institute of Child Health, London) was added at 500 units/ml to Hela cells at 50% confluence. The cells were harvested 24 and 48 hours later. From each cell culture, 0.5 x 10<sup>6</sup> cells were used for flow cytometry, and  
10 0.5 x 10<sup>6</sup> for reverse transcriptase-polymerase chain reaction (RT PCR) studies.

#### Monoclonal antibodies

The 16-27-12 (previously called NFKI) mouse IgG1 monoclonal  
15 antibody to HLA-DR was the kind gift of Dr S Fuggle (Oxford) (21), and the W6/32 mouse IgG1 monoclonal antibody to human class I MHC antigens was the kind gift of Professor AF Williams (Oxford) (22). The control F16-4-4 mouse IgG1 monoclonal antibody to rat class I MHC antigens has  
20 previously been described in detail (23).

#### Flow cytometry

All procedures were at 4<sup>0</sup>C or on ice, unless otherwise stated. Hela or DoHH2 cell suspensions were washed twice in  
25 0.10% bovine serum albumin (BSA) in phosphate buffered saline (PBS), and resuspended to 2 x 10<sup>7</sup> cells/ml in 0.5% BSA/PBS.

To 50 $\mu$ l of the cell suspension (10<sup>6</sup> cells) was added 50 $\mu$ l of  
30 saturating concentrations of monoclonal antibodies diluted in 0.5% BSA/PBS. After 30 minutes incubation, the cells were washed twice as above, and to the pellet of the second wash was added 50 $\mu$ l of saturating concentrations of fluorescein labelled rabbit anti-mouse immunoglobulin G  
35 (Dako, High Wycombe, UK) diluted in 0.5% BSA/PBS. The cells were resuspended, incubated for a further 30 minutes, and

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then washed twice as above. The pellet of the second wash was resuspended in 1ml of 2% formalin in PBS and 5000 cells were analysed at room temperature in a Facscalibur flow cytometer (Becton Dickinson, New Jersey, USA).

5

The data were analysed on CellQuest software (Becton Dickinson, New Jersey, USA). Cells were initially analysed using forward and right angle scatter. With both Hela and DoHH2 cells, the large majority of cells formed a tight  
10 cluster which was gated for the fluorescence studies.

The percentage suppression of MHC class II expression was calculated on the basis of the mean channel of fluorescence for class II of the test cells, standardised to the profile  
15 of the test cells with the negative control antibody as the zero point. The mean channel of fluorescence of interferon gamma treated normal cells was taken as 100%. For example, with clone 2 in Figure 3, the mean channels of fluorescence with the control antibody and the antibody to HLA-DR were  
20 2.6 and 4.9 respectively. The corresponding figures for the control Hela clone were 2.7 and 88.1. The percentage suppression was calculated as

$$100 \times [1 - (4.9 - 2.6) \div (88.1 - 2.7)] = 97.30\%.$$

#### 25 Construction of CIITA deletion mutants (Figure 1)

The full sequence of the CIITA cDNA present in pBlueScript (5) (kindly provided by Dr V Steimle, Geneva) was moved to the pcDNA3 expression vector using EcoRI and XhoI.

##### (a) pcDNA3mutCIITA2 (Fig. 1A)

30 This was constructed in 2 steps. First, the pcDNA3 CIITA construct was cut with NotI and XhoI, and the resulting fragment (representing position 1340 to the 3' end of the CIITA) was subcloned into NotI and XhoI sites of pcDNA3. Next, PCR was used to synthesise the fragment from amino  
35 acid 152 to the NotI site. The upstream primer,

5'ACTCGATATCATTTCCGGCAGACCTGAAGCAT3'

SEQ.ID.NO.5

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contained an introduced EcoRV site (underlined) at position 567 of the original sequence, while the downstream primer, 5'GCTCACTGCCCCAGCCCAATA3' SEQ.ID.NO.6

was complementary to the CIITA sequence immediately downstream of the NotI site. The amplified product was cut with EcoRV and NotI, purified on Chromaspin+TE-100 column (Clontech Lab, Inc, USA) and inserted at the EcoRV and NotI sites, resulting in pcDNA3mutCIITA2. Following insertion into pcDNA3, the PCR product was sequenced between the EcoRI and NotI sites, and confirmed to correspond exactly to the published sequence (5). This construct was used as a control since it lacked an initiation codon and would not give rise to CIITA protein.

(b) pcDNA3mutCIITA3 (Fig. 1B)

In order to support translation of the mutated cDNA, an oligonucleotide containing an initiation codon, followed by 5 codons corresponding to amino acids 2-6 of native CIITA, followed by a codon for isoleucine, was incorporated at the 5' end of the pcDNA3mutCIITA2 construct.

Two complementary oligonucleotides were synthesised containing the ATG initiation codon (under-lined) on the sense strand, an EcoRI site at the 5' end of the sense strand, and a blunt end at the other as follows:

5'AATTCTACACAATGCGTTGCCTGGCTCCA3' SEQ.ID.NO.7

5'TGGAGCCAGGCAACGCATTGTGTAG3'. SEQ.ID.NO.8

These were annealed to each other by heating at 95<sup>0</sup>C followed by cooling to room temperature over 3 hours. The annealed oligonucleotides were ligated into pcDNA3mutCIITA2 at the EcoRI and EcoRV sites. At the junction of this oligonucleotide with the remainder of the construct, as a consequence of this manipulation, the native codon for a leucine at position 151 was replaced by a codon for isoleucine. The rest of the sequence, ie from the codon for amino acid 152 to the end of the 3<sup>1</sup>-UT region, is as shown in Figure 5 and SEQ.ID.NOS.1 and 2. This was

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pcDNA3mutCIITA3.

(c) pcDNA3mutCIITA4 (Fig. 1B)

In order to ensure transport of the mutated CIITA molecule to the nucleus, another synthetic oligonucleotide construct was made. This was similar to that for pcDNA3mutCIITA3, but contained in addition 21 nucleotides encoding a nuclear localisation signal. The complementary oligonucleotides were:

5'AATTCTACACAATGCGTTGCCTGGCTCCAAAGAAGAAGCGCAAGGTC3'

10 SEQ.ID.NO.9

5'GACCTTGCGCTTCTTCTTTGGAGCCAGGCAACGCATTGTGTAG3' SEQ.ID.NO.10

This pair of oligonucleotides contained (in addition to the ATG initiation codon, the Eco RI site and the blunt end) the nuclear localisation signal (NLS) of the SV40 large T antigen (Lys Lys Lys Arg Arg Lys Val) (19,20). These were ligated into pcDNA3mutCIITA2 at the EcoRI and EcoRV sites, resulting in pcDNA3mutCIITA4.

(d) pCEP4 vectors (Fig. 1C)

20 The pcDNA3mutCIITA 2,3 and 4 vectors were cut by EcoRI, treated with Klenow large fragment DNA polymerase to create blunt ends, and subsequently cut with XhoI. These fragments were purified using QiaexII Gel Extraction (Qiagen, Germany) and cloned into the expression vector pCEP4 in the PvuII and  
25 XhoI sites, to create respectively pCEP4mutCIITA2,3 and 4.

#### DNA sequence analysis

The pcDNA3mutCIITA 2,3 and 4 constructs were sequenced between the EcoRI and NotI sites, using four oligonucleotide  
30 primers. One primer was complementary to the sequence of the pcDNA3 CMV promoter, 5'ATACGACTCACTATAGG3'. SEQ.ID.NO.11 The other 3 primers were complementary to the original CIITA cDNA sequence (Steimle et al., 1993) from positions 819 to 838, 1002-1026 and 1180-1208. 10 $\mu$ l plasmid DNA (1.5 $\mu$ g/ $\mu$ l)  
35 was subjected to sequencing reactions and run on an A.L.F.<sup>TM</sup> System (Pharmacia Biotech, Sweden) according to the

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manufacturer's instructions.

#### Transfections

Hela and DoHH2 cells were washed twice in OPT1-MEMI serum-free medium (Gibco BRL, Paisley, UK), and  $1 \times 10^6$  cells in  
5 0.8ml of OPT1-MEMI were seeded into each well of 6 well tissue culture plates (Falcon or Becton-Dickinson, New Jersey, USA). For the transfections,  $3\mu\text{g}$  of DNA construct and  $10\mu\text{g}$  of lipofectamine (Gibco BRL, Paisley, UK) were mixed in  $200\mu\text{l}$  of OPT1-MEMI and incubated for 30 minutes at room  
10 temperature to allow DNA-lipofectamine complexes to form. This was added to the appropriate well on the tissue culture plate, mixed gently to ensure uniform distribution and incubated for 5 hours at  $37^\circ\text{C}$  in 95% air/5%  $\text{CO}_2$ . At the end of this incubation, 4ml of the appropriate medium with 12.5%  
15 FCS was added to each well.

At 48 hours after transfection, the Hela cells were passaged into medium containing  $500\mu\text{g/ml}$  G418 (Gibco BRL, Paisley, UK)

The medium was changed every 2 days. Colonies were picked  
20 after 2-4 weeks in the selective medium, and were maintained in flasks in selective medium.

The DoHH2 cells (which grow as a suspension culture) were taken 48 hours after transfection and maintained with  $500\mu\text{g/ml}$  of G418 in the medium.

25

#### Semi-quantitative RT-PCR analysis

Messenger RNA was prepared from Hela and DoHH2 cells using an mRNA Purification System (Pharmacia Biotech, Uppsala, Sweden), and the amount of mRNA recovered was established  
30 spectrophotometrically using a GeneQuant (Pharmacia Biotech, Uppsala, Sweden). Complementary DNA was synthesised using 320ng mRNA and the First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The solution was denatured at  
35  $95^\circ\text{C}$  for 5 minutes and different amounts of template (equivalent to 200ng, 50ng, 10ng, 2ng and 0.4ng mRNA) were

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amplified in the presence of primers specific for actin, endogenous CIITA, HLA-DRA and mutated CIITA, as follows:  
HLA-DRA chain:

upstream primer: 5'CGAGTTCTCTATCTGAATCCTG3' SEQ.ID.NO.12

5 (From exon 1)

downstream primer: 5'GTTCTGCTGCATTGCTTTTGC3' SEQ.ID.NO.13

(From exon 2)

endogenous CIITA:

upstream primer: 5'ACTCCGGGAGCTGCTGCCCTGGC3' SEQ.ID.NO.14

10 downstream primer: 5'CCTGGAAGACATACTGGTCC3' SEQ.ID.NO.15

transfected CIITA:

upstream primer: 5'AATTCTACACAATGCGTTGCCTGGCTCCA

SEQ.ID.NO.16

downstream primer: 5'GTTGGGAGGCCGTGGACAGTG

SEQ.ID.NO.17

15 actin:

upstream primer 5'GGGCATGGGTCAGAAGAATT3' SEQ.ID.NO.18

(From exon 5)

downstream primer 5'TACATGGCTGGGGTGTGAA3' SEQ.ID.NO.19

(From exon 7)

20

The upstream primer for the transfected CIITA is from the inserted synthetic oligonucleotide. It therefore cannot recognise endogenous CIITA but is specific for mutCIITA3 and mutCIITA4. The upstream primer for endogenous CIITA is from  
25 the regions deleted in all mutant constructs. It therefore cannot recognise the mutated CIITAs, but is specific for endogenous CIITA.

## RESULTS

### Construction of deletion-mutants of the human CIITA molecule

30 Our objective was to remove the smallest portion of the amino terminal region which, would completely abolish activation of transcription, in order to give the optimal chance for the remainder of the molecule to retain its native conformation. We chose to remove the first 151 amino  
35 acids (bases 1-566) in the first instance. The overall plan was to remove more or fewer amino acids, depending on the

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characteristics of these initial constructs, but this proved not to be necessary.

As described in detail in Materials and Methods,  
5 pcDNA3mutCIITA2 was constructed in 2 steps, involving first the transfer of a subclone (bases 1340-4543) of the full length cDNA, followed by a PCR product encompassing bases 567-1340 (Fig.1a.) Following insertion into pcDNA3, the PCR product was sequenced between the CEORI and NotI sites, and  
10 confirmed to correspond exactly to the published sequence (5). This construct was used as a control since it lacked an initiation codon and would not give rise to CIITA protein.

15 In order to support translation of the mutated cDNA, an oligonucleotide containing an initiation codon, followed by 5 codons corresponding to amino acids 2 to 6 of native CIITA followed by a codon for isoleucine was incorporated at the 5' end of the construct. At the junction of this  
20 oligonucleotide with the remainder of the construct, as a consequence of this manipulation, the native codon for a leucine at position 151 was replaced by a codon for isoleucine. The remainder of the sequence was the same as human native CIITA, including the 3<sup>1</sup>UT sequence.

25 In order to ensure transport of the mutated CIITA molecule to the nucleus, another synthetic oligonucleotide construct was made. This was similar to that for pcDNA3mutCIITA3, but contained 21 nucleotides encoding a nuclear localisation  
30 signal. This was pcDNA3mutCUUTA4.

All 3 mutated CIITA constructs were placed in the expression vectors pcDNA3 and pCEP4, in both of which transcription is controlled by the CMV promoter (Fig.1C). The pCEP4 vector  
35 has the potential to support episomal growth in human cells, via an EBV origin of replication.



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Base-line studies

RT-PCR experiments demonstrated that neither CIITA nor HLA-DR $\alpha$  mRNA could be detected in Hela cells prior to stimulation with interferon gamma. However, these were readily detectable within 24 hours of stimulation (Fig.2A, lanes 1 and 2). By contrast, the DoHH2 cell line had readily detectable mRNA for both CIITA and HLA-DRA, which was not obviously influenced by exposure to interferon gamma (Fig.2A, lanes 3 and 4).

In agreement with the mRNA data, HLA-DR protein was readily detectable by flow cytometry after, but not before, interferon gamma stimulation of Hela cells (Fig.2B, panel 1). Hela cells strongly express MHC class I molecules, which are further upregulated by interferon gamma (Fig.2B, panel 2). This provides an excellent control for the class II expression studies. As expected, the DoHH2 cell line strongly and constitutively expressed both HLA-DR and class I molecules (Fig.2B, panel 3), and these were not upregulated by interferon gamma (data not shown).

Suppression of HLA-DR induction in Hela cells by mutated CIITA(a) pCDNA3 expression vector

Initial studies involved transfection of Hela cells with each of the 3 mutant CIITA constructs in the pCDNA3 vector, and the establishment of stable, transfected clones. In the order of 50-100, generally between 27 and 95 stable Hela clones were established for each mutant construct, and each clone was screened for class II MHC expression before and after treatment with interferon gamma, using flow cytometry.

A minimum of 10 clones for each construct was examined for HLA-DRA, CIITA and mutant CIITA mRNA expression by semi-quantitative RT-PCR.

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All 28 clones transfected with the empty pcDNA3 vector, and all 27 clones transfected with the control CIITA construct without the initiation codon (pcDNA3mutCIITA2) had normal class II MHC induction by flow cytometry. In excess of 10 clones with each construct were also tested by semi-quantitative RT-PCR and all gave essentially the same result. A representative clone transfected with pcDNA3mutCIITA2 and analysed by flow cytometry (Fig.3B, clone 1) and by semi-quantitative RT-PCR (Fig.3A, clone 1) showed normal class II and class I MHC antigen induction, as well as normal induction of mRNA for CIITA and HLA-DRA.

Thirty six of 62 Hela clones transfected with pcDNA3mutCIITA3 (with initiation codon) and 54 of 95 Hela clones transfected with pcDNA3mutCIITA4 (with initiation codon and nuclear localisation signal) showed clear (>35%) suppression of class II MHC induction. Nineteen of the 36 and 22 of the 54 clones showed very strong suppression of MHC class II induction and were chosen for further study. Flow cytometry profiles for representative clones are shown in Fig. 3B, clones 2 and 3, with semi-quantitative RT-PCR analysis on these same cultures in Fig.3A, clones 2 and 3.

The flow cytometry demonstrated a down regulation in mean fluorescence for class II of 97% (previously 55% with pcDNA3mutCIITA3, and (previously 60%) 98% with pcDNA3mutCIITA4, without any reduction in class I expression. The RT-PCR studies showed normal induction of endogenous CIITA mRNA in clones 2 and 3 but, in spite of this, a substantial (~20 fold) suppression of HLA-DRA mRNA expression. There was no consistent advantage pcDNA3mutCIITA4 (with initiation codon and nuclear localisation signal) over pcDNA3mutCIITA3 (with initiation codon only).

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None of the Hela clones expressing the pcDNA3mutCIITA3 or 4 constructs showed any expression of class II antigens prior to interferon gamma stimulation (Figure 3B, clones 2 and 3). Thus, removal of the amino terminal 151 amino acids abolished transcription activation by CIITA even though the truncated CIITA molecules could presumably bind well to the promoter proteins to compete effectively with native CIITA. Expression of mutant CIITA mRNA was clearly visible (Fig.3A, clones 2 and 3). Omission of the reverse transcriptase step resulted in no PCR products (data not shown), formally demonstrating that the mutated CIITA PCR product was derived from expressed mRNA, and not genomic DNA or surviving plasmid DNA. The relative expression of endogenous and mutant CIITA cannot be assessed from the RT-PCR studies, as the efficiency of the primers for PCR could vary for the two molecules.

(b) pCEP4 expression vector

Because of the possibility of higher levels of expression of mutated CIITA from multiple episomal copies, the vector pCEP4 was also used. Transiently transfected Hela cell cultures were used, but hygromycin selection was applied to remove non-transfected cells.

Hela cells were transfected with the control mutCIITA2 and the mutCIITA4 constructs in pCEP4. Five to 11 days after selection with hygromycin, the bulk cultures were stimulated with interferon gamma and subjected to flow cytometry and semi-quantitative RT-PCR analysis. The experiment was repeated on 11 occasions. The control cultures transfected with the empty pCEP4 vector (data not shown) or the control pCEP4mutCIITA2 construct (Fig.3B, culture 1 and Fig.3A, culture 1) showed the expected normal pattern of MHC class I and class II protein expression, and CIITA and HLA-DRA mRNA expression. However, on 7 of the 11 occasions, the cultures transfected with pCEP4mutCIITA4 constructs showed >60% suppression of class II MHC expression, the suppression

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being >93% in 4 of these 7 cultures. The results for one of the transfections with the mutCIITA4 construct are given in Fig.4A and B, culture 2. The down-regulation in mean fluorescence for class II was 99% for this culture, and HLA-DRA mRNA was substantially reduced (~20 fold). Transfection of the mutant CIITA genes did not affect the induction of endogenous CUUTA mRNA by interferon gamma in any of the cultures. However, HLA-DR $\alpha$  mRNA was reduced, either partially as shown in Fig. 3A, culture 2, or almost completely in some cultures. Expression of the mutant CIITA construct was readily visible.

Suppression of constitutive HLA-DR expression in a B lymphoblastoid cell line by mutated CIITA

The DoHH2 cell line was transiently transfected with each of the pcDNA3mutCIITA constructs, but G418 selection was applied to remove non-transfected cells. At days 5 and 8 following selection with G418, the cultures were examined by flow cytometry and semi-quantitative RT-PCR. The experiment was repeated on 20 occasions. For any particular experiment, the results at days 5 and 8 were very similar. The flow cytometry studies showed no suppression of class I MHC expression at any stage in any of the cultures (data not shown). Flow cytometry studies for MHC class II expression are given in Fig. 4B. Cultures transfected with pcDNA3 alone (culture 1), or pcDNA3mutCIITA2 (culture 2) (data not shown) showed no reduction in class II expression. Cultures transfected with pcDNA3 alone, or pcDNA3mutCIITA2 showed no reduction in class II expression. However, cultures transfected with pcDNA3mutCIITA3 (Fig.4B, panel 1) or pcDNA3mutCIITA4 (Fig.4b, panel 2) showed a reduction of approximately 45% in the mean fluorescence for class II. given that the half-life of class II MHC molecules on the cell surface is of the order of 4 days (24), this potentially represents a strong suppression of de novo class II synthesis. This was in fact suggested by the RT PCR data

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(Fig.4a). In none of the cultures was there any suppression of endogenous CIITA mRNA expression. However, in the pcDNA3mutCIITA3 and pcDNA3mutCIITA4 transfected B cells, HLA-DR $\alpha$  mRNA was not detectable.

5

## DISCUSSION

The original description of the cDNA sequence of CIITA by Steimle et al. (5) suggested that the amino terminal region of this molecule might be involved in the activation of transcription of MHC class II genes. Subsequent studies by Riley et al. (13) and Zhou and Glimcher (14) established that this was indeed the case. Zhou and Glimcher (14) also demonstrated that the carboxy terminal 830 amino acids could specifically direct transcription from the HLA-DR $\alpha$  promoter, although less efficiently than the full length 1130 amino acid CIITA molecule. However, this 830 amino acid fragment was unable to suppress constitutive MHC class II expression.

Here we demonstrate that removal of the amino terminal 151 amino acid acidic domain leaves a truncated protein of 979 amino acids which inhibits both constitutive and interferon gamma induced MHC class II expression.

The three proline/serine/threonine rich domains, which are found at amino acid positions 163-322 in the native CIITA protein, are intact in our construct, but missing from that of Zhou and Glimcher (14). It would therefore seem likely that this region of CIITA either plays a critical role in the conformation of the remainder of the protein, or is itself directly involved in critical protein/protein interactions.

Our mutated CIITA proteins might function as dominant negative suppressors by competing effectively with endogenously produced CIITA for binding to proteins in the promoter region of MHC class II genes. However, the

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relative expression of endogenous CIITA mRNA and mutant CIITA mRNA in the transfected cell lines cannot be assessed from our RT-PCR studies, as the efficiency of the primers for PCR is likely to vary for the two molecules. Whereas a  
5 long strand of sequence was available to select the optimal upstream primer specific for endogenous CIITA, that for mutant CIITA was restricted to the inserted oligonucleotide containing the initiation codon. Nevertheless, it is worth noting that transcription activators frequently are active  
10 as dimers or higher multimers (e.g. 16,25,26). If this is the case for CIITA, it is possible that in addition to competing for binding to proteins in the promoter region, the mutated proteins either are unable to form multimers, or that dimers or multimers incorporating a mutant CIITA  
15 molecule are functionally compromised. In these circumstances the suppressive effect of mutated CIITA molecules would be greater than would be expected from the relative concentrations of endogenous and mutated forms.

20 Although the region of the CIITA protein important for nuclear localisation is not known, our studies suggest that it is not present in the N terminal 151 amino acids. This can be inferred from the equal effectiveness of mutant constructs, irrespective of whether or not a known nuclear  
25 localisation signal is added. As nuclear localisation signals tend to be basic (26), one would in fact not expect this signal to be in the acidic region of the molecule.

The suppression of MHC class II expression by these mutant  
30 CIITA proteins has potentially important applications for regulating clinically relevant immune responses, especially in autoimmunity and transplantation. For example, it has been suggested that class II MHC expression on vascular endothelial cells plays a critical role in the long-term  
35 immunogenicity of transplanted human organs (28). The suppression of this expression might substantially reduce

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the incidence of chronic rejection and the requirement for long-term immunosuppression. In the pig-to-human xenograft system, it is now well established that human T cells can respond directly to porcine MHC class II molecules (29) and these molecules therefore present potentially important targets for immune regulation. It is important to note, from studies down-regulating MHC expression in B cells by antisense oligonucleotides, that partial suppression of MHC expression on antigen presenting cells can have major effects on the efficacy of antigen presentation.

For experimental studies, the in vivo down-regulation of class II expression has been possible by the use of the gene knock-out approach, in particular for CIITA (30). These approaches give rise to global suppression of class II MHC genes. Our construct would allow tissue-specific down-regulation, by the generation of transgenic mice carrying the mutant CIITA genes under tissue specific promoters.

## 20 EXAMPLE 2

### Method:

A hammerhead ribozyme targeted against bases 1159-1161 (GUA) of human CIITA mRNA was constructed according to the methods described in Tanner et al (24) and in Larsson et al (32).

The antisense arms of the ribozyme are

5' TGTTGGA \* ribozyme \* ACGTGC 3'.

Hammerhead ribozymes were also targeted against other regions of the CIITA mRNA.

The ECV-40 human vascular endothelial cell line maintained in Medium 199, non HEPES with 10% foetal calf serum, and the Hela cell line maintained in DMEM were transfected with the

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various ribozyme constructs or with an empty construct and then exposed 500 or 1000 units of human interferon gamma to induce class II MHC expression, as described for Hela cells in Example 1. Using flow cytometry as described in Example 1, MHC induction was measured 24, 48 and 72 hours after the addition of the interferon.

#### Results:

The ribozyme targeted at bases 1159-1161 markedly suppressed MHC induction, whereas the empty construct and the other ribozymes were without effect. Figure 6A shows class II MHC induction in ECV-40 cells at 24, 48 and 72 hours using 500 and 1000 units of human interferon gamma. Figure 6B shows induction in ECV-40 cells transformed with the ribozyme.

The ribozyme markedly suppresses the induction of class II MHC expression. Suppression of class II MHC expression is also shown in Figure 7, which demonstrates levels of expression in untreated ECV-40 cells and in non-transfected and ribozyme-transfected ECV-40 cells stimulated with interferon. Class II MHC expression was determined 72 hours after interferon stimulation.

#### Discussion:

The suppression of class II expression by the ribozyme targeted at bases 1159-1161 has the same potentially important clinical applications for the ribozyme in regulating clinically relevant immune responses, especially in autoimmunity and transplantation as does the suppression by the deletion mutant CIITA polypeptide.

#### EXAMPLE 3

##### **Materials and Methods**

##### Construction of CIITA deletion mutants

The pcDNA3mutCIITA2 construct, the pcDNA3mutCIITA3 construct and the pcDNA3mutCIITA4 construct were produced as described in Example 1.



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DNA sequence analysis

The pcDNA3mutCIITA 2, 3 and 4 constructs were sequenced between the EcoRI and NotI sites to confirm the sequence of the PCR product. Ten  $\mu$ l plasmid DNA ( $1.5\mu\text{g}/\mu\text{l}$ ) was subjected to sequencing reactions and run on an A.L.F.<sup>TM</sup> System (Pharmacia Biotech, Sweden) according to the manufacturer's instructions.

The porcine CIITA fragment was sequenced directly from the RT-PCR product from L23 cells, using a fluorescein-labelled primer and the sequenase fluorescent labelled primer cycle sequencing kit (Amersham International, Amersham, Bucks, UK).

Cell lines

The porcine B cell line L23<sup>17</sup> was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts, UK). It was maintained in RPMI 1640 supplemented with 10% foetal calf serum (FCS) (Gibco, Paisley, UK), 2mM glutamine, 1mM Sodium pyruvate, 50 $\mu$ M 2-mercaptoethanol, 150 units/ml of penicillin and 150 $\mu$ g/ml of streptomycin. The porcine vascular endothelial cell line PIEC<sup>18</sup> was a kind gift from Dr. K. Welsh (Oxford, UK) and was maintained in RPMI 1640 with 10% FCS, 2mM glutamine, 150 units/ml of penicillin and 150  $\mu$ g/ml of streptomycin.

Transfections

L23 and PIEC cells were washed twice in OPTIMEM I serum-free medium (Gibco, Paisley, UK) and  $1 \times 10^6$  cells in 0.8ml of OPTIMEM I were seeded into each well of 6 well plates (Becton-Dickinson Labware Europe, Meylan, France).

Transfection complexes were formed by mixing 3 $\mu$ g of the DNA construct and 10 $\mu$ g of lipofectamine (Gibco, Paisley, UK) in 200 $\mu$ l of OPTIMEM I. This was incubated for 30 minutes at

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room temperature to allow DNA-lipofectamine complexes to form, and then added to the cells. The cells were incubated for 5 hours at 37°C in 5% CO<sub>2</sub>/95% air, after which 4ml of the appropriate growth medium with 12.5% foetal calf serum was added to each well.

After 48 hours, the cells were passaged into selective medium containing 500 $\mu$ g/ml of G418 (Gibco, Paisley, UK). The medium was changed every 2 or 3 days. Stable PIEC clones were selected after a minimum of 3 weeks in the selective medium, and thereafter maintained in selective medium.

#### Treatment with interferon gamma

Recombinant porcine interferon  $\gamma$  (rpoIFN $\gamma$ )<sup>19</sup> was a kind gift of Dr. R. Steiger (Ciba-Geigy, Basel, Switzerland). PIEC cells in 6 well plates were cultured for 72 hours with 800 units/ml of rpoIFN $\gamma$ . G418 was not added to the medium during rpoIFN $\gamma$  treatment. At the end of the 72 hr incubation, the cells were harvested by vigorous pipetting, and divided into aliquots for flow cytometry, reverse transcriptase-polymerase chain reaction (RT-PCR) and T cell proliferation studies.

#### Mouse monoclonal antibodies

The H42A IgG2a antibody to SLA-DQ antigens, the MSA3 IgG2a antibody to SLA-DR antigens and the PT85A IgG2a antibody to SLA class I antigens were all purchased from VMRD (Pullman, WA). The mouse IgG1 antibody to human CD4 (MCA1267F) was purchased from Serotec (Bicester, Oxon, UK). The control F15-42-1 IgG1 antibody to human Thy-1 has been described previously<sup>20</sup>.

#### Flow cytometry

All procedures were at 4°C or on ice, unless otherwise stated. PIEC or L23 cell suspensions were washed twice by

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centrifugation in 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and resuspended to  $2 \times 10^7$  cells/ml in 0.5% BSA/PBS. To 50  $\mu$ l of the cell suspension ( $10^6$  cells) was added 50  $\mu$ l of saturating concentrations of monoclonal antibody diluted in 0.5% BSA/PBS. After 30 min incubation, the cells were washed twice and to the pellet of the second wash was added 50  $\mu$ l of saturating concentrations of fluorescein-labelled rabbit anti-mouse IgG (Dako, High Wycombe, UK) diluted in 0.5% BSA/PBS. The cells were resuspended, incubated for a further 30 min and then washed twice. The pellet of the second wash was resuspended in 1 ml of 2% formalin in PBS and 5000 cells were analysed at room temperature in a FACScalibur flow cytometer (Becton Dickinson, San Jose, California, USA).

The data were analysed on CellQuest software (Becton Dickinson). Cells were initially analysed using forward and right angle scatter. With both PIEC and L23 cells, the large majority of cells formed a tight cluster which was gated for the fluorescence studies. The percentage suppression of MHC class II expression was calculated on the basis of the mean channel of fluorescence for class II of the test cells, with the profile of the test cells with the negative control antibody as the zero point. The mean channel of fluorescence of IFN- $\gamma$  treated normal cells was taken as 100%. For example, with clone 4 in Fig. 5, the mean channels of fluorescence with the control antibody and the antibody to SLA-DR were 1.9 and 2.1 respectively. The corresponding figures for the control PIEC clone were 1.9 and 110.8. The percentage suppression was calculated as:

$$100 \times [1 - (2.1 - 1.9) \div (110.8 - 1.9)] = 99.8\%.$$

#### Semi-quantitative RT-PCR analysis

Messenger RNA was prepared from PIEC and L23 cells using an mRNA Purification System (Pharmacia Biotech, Cambridge, UK).

The amount of mRNA recovered was established

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spectrophotometrically using a GeneQuant (Pharmacia Biotech). Complementary DNA was synthesized using approximately 1 µg of mRNA and the First-Strand cDNA Synthesis Kit (Pharmacia Biotech) according to the manufacturer's instructions. The solution was denatured at 95°C for 5 min and different amounts of template (equivalent to 50, 10, 2 and 0.4ng of mRNA) were amplified in the presence of primers specific for actin, endogenous porcine CIITA, SLA-DRA and mutated human CIITA as follows:

## SLA-DRA chain:

upstream primer: 5'-GATCAAGCGCTCCAACAACACC-3' SEQ.ID.NO.20  
(from exon 1)

downstream primer: 5'-GATGCCCACCAGAGCCACAAT-3' SEQ.ID.NO.21  
(from exon 2)

## Endogenous porcine CIITA:

upstream primer: 5'-TACACAATGCGTTGCCTGGCTCCA-3' SEQ.ID.NO.22

downstream primer: 5'-CCTGGAAGACATACTGGTCC-3' SEQ.ID.NO.23

## Transfected human CIITA:

upstream primer: 5'-AATTCTACACAATGCGTTGCCTGGCTCCA-3' SEQ.ID.NO.16

downstream primer: 5'-GTTGGGAGGCCGTGGACAGTG -3' SEQ.ID.NO.17

## Actin:

upstream primer: 5'-GGGCATGGGTCAGAAGGATT-3' SEQ.ID.NO.18  
(from exon 3)

downstream primer: 5'-TACATGGCTGGGGTGTGAA-3' SEQ.ID.NO.19  
(from exon 4)

The upstream primer for endogenous porcine CIITA is from the region deleted in all mutated human constructs. It therefore cannot recognize the mutated human CIITA, and is specific for endogenous porcine CIITA in the pig cells transfected with mutated human CIITA. (However, the primer cannot distinguish full length porcine from full length

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human CIITA).

The upstream primer for the transfected human CIITA is from the inserted synthetic oligonucleotide. It therefore cannot  
5 recognize endogenous porcine (or human) CIITA, but is specific for mutCIITA3 and mutCIITA4.

#### Preparation of human CD4+ T lymphocytes

Peripheral blood mononuclear cells (PBLs) were prepared from  
10 ~60ml of EDTA anticoagulated blood by centrifuging for 25 min at 600g at room temperature with Nycoprep (Nycomed Pharma, Oslo, Norway). The PBLs were washed twice by centrifugation and resuspended in 1.5ml of 15mM Hepes buffered RPMI 1640 medium (Gibco) with 1% FCS.

15 To remove adherent cells, the 1.5ml of PBL was applied to a column containing 10-12ml of sterile G10 beads (Pharmacia, Uppsala, Sweden) previously equilibrated in Hepes buffered RPMI 1640 medium with 1% FCS at 37°C. After 45 minutes at  
20 37°C, the non-adherent cells were eluted with 10-20 ml of medium, centrifuged, and resuspended in 1.5ml. This was applied to a second G10 column as above, to ensure full removal of adherent cells. The cells were then centrifuged and resuspended to  $4 \times 10^7$  cells/ml in the above medium.

25 Positive selection of CD4+ T cells was performed with anti-CD4 coated Dynabeads (DynaI, Oslo, Norway). Seven hundred  $\mu$ l ( $1.6 \times 10^8$ ) of beads were washed 5 times in Hepes buffered RPMI 1640 with 1% FCS, using a magnet, and 1ml of PBL  
30 containing ~  $4 \times 10^7$  cells was added (giving a ratio of ~8 beads/CD4+ T cell). This was incubated at 4°C for 1 hour, with gentle end-over-end mixing. The beads and attached cells were washed 5 times using the magnet, and then resuspended in 120 $\mu$ l of medium. Twelve  $\mu$ l (1.2 units) of  
35 detach-a-bead (DynaI) was added, and incubated at room temperature for 45 minutes with gentle agitation to separate

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the cells. The detached cells were washed twice and resuspended to  $2.5 \times 10^6$  CD4+ T cells/ml in RPMI 1640 with 10% FCS, 2mM glutamine, 150 units/ml of penicillin and 150  $\mu$ g/ml of streptomycin.

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Viability of the CD4+ T cells was >90% by trypan blue exclusion and the purity always > 99% as assessed by flow cytometry with the monoclonal antibody to CD4.

10 T cell proliferation assays

The PIEC cells were washed three times in fresh medium, irradiated with 3,500 rads (IBL437C Irradiator, CIS Biointernational), and harvested by vigorous pipetting.

Five  $\times 10^4$  PIEC cells in 100 $\mu$ l of culture medium were added to individual wells of U-bottomed 96 well plates (Falcon, Becton-Dickinson). These were cultured overnight and  $2.5 \times 10^5$  CD4+ T cells in 100 $\mu$ l of culture medium were added in triplicate. Cultures were harvested after 3, 4, 5 and 6 days, the wells being pulsed with 1 $\mu$ Ci of  $^3$ H-thymidine 18 hours before harvest. Cell bound  $^3$ H thymidine was counted on a MicroBeta counter (EG and G Berthold, Milton Keynes, UK).

Monocyte contamination was assessed by culturing with 5 $\mu$ g/ml of phytohaemagglutinin (PHA) for 3 days.

**RESULTS**

Construction of deletion-mutants of the human CIITA molecule (Fig. 8)

30 Our objective was to remove the smallest portion of the N-terminal region which would completely abolish activation of transcription, in order to give the optimal chance for the remainder of the molecule to retain its native conformation.

We chose to remove the first 151 amino acids (bases 1-566) in the first instance. All three mutated CIITA constructs were placed in the expression vector pcDNA3, in which

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transcription is controlled by the CMV promoter.

As shown in Example 1, pcDNA3mutCIITA3 and pcDNA3mutCIITA4 constructs can suppress both constitutive and interferon gamma induced MHC class II expression in human cell lines<sup>14</sup>.

The presence (in mutCIITA4) or absence (in mutCIITA3) of the NLS made no difference to the effectiveness of these constructs in human cells. The control mutCIITA2 construct (lacking an initiation codon for the translation of the mRNA) was without effect.

#### Activity of human CIITA in porcine cells

Human CIITA is able to induce MHC class II antigens in the mouse<sup>21</sup>. The mutated human constructs was examined in the pig, because of the potential importance of this species in clinical xenotransplantation.

The easiest way to assess cross-species reactivity was to transfect the PIEC cell line with the full length human CIITA construct. The results are given in Figure 9. The PIEC cell line (Fig. 9A, top profile) normally expresses neither SLA-DR nor SLA-DQ antigens, but strongly expresses SLA class I antigens. Both transient (Fig. 9A, middle profile) and stable (Fig. 9A, lowest profile) PIEC transfectants strongly express both SLA-DR and SLA-DQ antigens. SLA-Class I antigen expression is unaffected in the CIITA transfectants. It is important to note that the level of expression of SLA-DR and SLA-DQ by human CIITA was similar to that induced by rpo IFN $\gamma$  (see later).

The RT-PCR studies (Fig. 9B) demonstrated that normal PIEC express neither CIITA nor SLA-DRA mRNA (lane 1), but that both transient (lane 2) and stable (lane 3) PIEC transfectants expressed SLA-DRA mRNA. Although the CIITA primers do not distinguish full length human from full length porcine CIITA, the CIITA mRNA seen in lanes 2 and 3

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presumably represents the human CIITA mRNA transgene product.

Having demonstrated the strong capacity of human CIITA to induce MHC class II antigens in porcine cells the effect of the mutated human CIITA construct was investigated.

#### Preliminary studies for the detection of porcine CIITA mRNA

Porcine CIITA cDNA has not yet been cloned and sequenced.

However, it was important to be able to detect porcine CIITA mRNA by PCR. Several primer pairs were tested, based on the human CIITA sequence, on cDNA derived from the L23 porcine B cell line. Several primer pairs gave products of the predicted size. One of these primer pairs, spanning bases 140 to 437 of the human sequence set out in Figure 5 and SEQ.ID.NO.1 was chosen for further study because the PCR product is derived from the region of human CIITA which is deleted in the mutated human constructs. These primers therefore distinguish endogenous porcine CIITA from the mutated human CIITA.

#### Baseline studies

Both flow cytometry (A) and RT-PCR studies were performed on the porcine endothelial cell line (PIEC) and the porcine B cell line (L23).

The results in Figure 10A (Upper and middle panels) demonstrate that treatment of the PIEC cell line with recombinant porcine interferon gamma strongly induced both SLA-DR and SLA-DQ antigens, and further increased expression of SLA class I antigens. Interestingly, the L23 porcine B cell line (Fig. 10A, lower panel) has much higher constitutive expression of SLA-DQ as compared to SLA-DR antigens and very high levels of SLA class I antigens. The RT-PCR studies in Fig. 10B show that the interferon gamma induced porcine CIITA and SLA-DRA mRNA in the PIEC line, and



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that the L23 B cell line expresses these mRNAs constitutively.

Suppression by mutated human CIITA of SLA-DQ and SLA-DR induction by interferon gamma

A series of stably transfected PIEC clones was established, using the empty pcDNA3 vector (24 clones), the control construct pcDNA3mutCIITA (24 clones), the experimental construct with the initiation codon pcDNA3mutCIITA3 (24 clones) and the experimental construct with both initiation codon and NLS pcDNA3mutCIITA4 (48 clones). All 120 clones were screened by flow cytometry for MHC class II antigen induction by recombinant porcine interferon gamma. All clones transfected with the empty vector or the control construct had normal SLA-DR and SLA-DQ induction, and representative flow cytometry profiles are shown in Fig. 5A (rows 1 and 2). All of the PIEC clones transfected with the pcDNA3mutCIITA3 construct also had normal SLA-DR and SLA-DQ induction, in spite of the fact that this construct was effective in human cell lines<sup>14</sup>. A representative flow cytometry profile is shown in Fig. 11A (row 3). The pcDNA3mutCIITA4, however, was highly effective. Of the 48 stable clones, 21 had normal class II induction, while 27 had definite ( $\geq 40\%$ ) suppression of induction. Of these, the majority (18 of the 27 clones) showed strong ( $\geq 90\%$ ) suppression. A representative clone showing  $>99\%$  suppression of SLA-DR and SLA-DQ induction is illustrated in Fig. 11A (bottom row). SLA-DR and SLA-DQ were equally suppressed in these clones, and there was no obvious effect on SLA class I expression. This level of effectiveness of pcDNA3mutCIITA4 on porcine PIEC cells was similar to that previously seen with human ECV3 cells<sup>14</sup>, the trend in fact being for greater effectiveness on porcine cells.

Semiquantitative RT-PCR studies are given in Fig. 11B. The clones transfected with the control constructs (lanes 1 and

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2) or pcDNA3mutCIITA3 (lane 3) showed normal induction of porcine CIITA and SLA-DRA mRNA. However, the pcDNA3mutCIITA4 transfected clone (lane 4) showed marked suppression of SLA-DRA mRNA in spite of normal levels of induction of porcine CIITA.

It is important to note that whereas the mutated human CIITA mRNA was readily detected in the pcDNA3mutCIITA4 clones (represented in lane 4), it was not detected in any of the pcDNA3mutCIITA3 clones (represented in lane 3). Omission of the reverse transcription step resulted in no PCR products (data not shown), formally demonstrating that the CIITA products were derived from mRNA, and not from genomic DNA or surviving plasmid DNA.

It is important also to note that one cannot infer the relative levels of endogenous porcine CIITA and mutated human CIITA mRNAs from these studies, as the efficiency of the primers for the two products is likely to be different.

This is particularly so as the upstream primer for the mutated human CIITA could be selected only from the short region of synthetic oligonucleotides inserted into pcDNA3mutCIITA3.

#### Suppression of constitutive SLA-DR and SLA-DQ expression in a porcine B cell line

The L23 cell line was transfected with the empty vector, and with the 3 mutated human CIITA constructs. After 2 days, selection medium was applied and the cells were analysed 3, 5, 7, 9 and 11 days later by flow cytometry and RT-PCR. In initial experiments, optimal suppression of MHC class II expression was found at days 5 and 7, and subsequent experiments were therefore performed at days 5 and 7 only. The experiment was performed on 12 occasions. On none of these occasions was there any suppression of MHC class II expression in cultures transfected with the empty pcDNA3

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vector or with the control pcDNA3mutCIITA2 construct. Similarly, as was to be expected from the results in the preceding section, pcDNAMutCIITA3 also had no effect. However, on 8 occasions, there was substantial (> 50%)  
5 suppression of MHC class II expression in cultures transfected with pcDNAMutCIITA4. The level of suppression was in the range of 50%-86% for SLA-DR and 55% to 92% for SLA-DQ. MHC class I expression was unaffected in these cultures. The flow cytometry studies are illustrated in  
10 Fig. 12A. For culture 4 (bottom row) the level of suppression of antigen expression was 86% for SLA-DR and 92% for SLA-DQ. It is worth noting again that the starting level of SLA-DQ in the L23 cells is much higher than the starting level of SLA-DR.

15 The RT-PCR studies (Fig. 12B) showed strong constitutive expression of porcine CIITA in all cultures. However, in spite of this, SLA-DRA mRNA was undetectable in the pcDNA3mutCIITA4 transfected cultures.

20 The failure of pcDNAMutCIITA3 to suppress constitutive MHC class II expression in the porcine L23 cell line was consistent with the failure of this construct to suppress IFN $\gamma$  induced porcine MHC class II suppression in PIEC cells, as reported in the preceding section. The important point  
25 to note is that, as with the stably transfected PIEC clones reported in the preceding section, mutated human CIITA mRNA was undetectable in the pcDNA3mutCIITA3 cultures (Fig. 12B, lane 3) but was readily detectable in its pcDNA3mutCIITA4  
30 cultures (Fig. 12B, lane 4). Omission of the reverse transcriptase step resulted in no PCR product (data not shown) formally demonstrating that the mutated CIITA product was derived from mRNA and not from genomic or residual plasmid DNA.

35 Suppression of the capacity of PIEC to stimulate direct T

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cell xenorecognition

Human CD4+ T cells were purified from peripheral blood and shown to be thoroughly depleted of APC by their failure to respond to PHA (Fig. 13, day 3).

The capacity of PIEC cells to stimulate direct recognition by human CD4+ T cells was tested using normal and interferon gamma stimulated PIEC. It can be seen that normal PIEC did not stimulate pure human CD4+ T cells (as expected), but that interferon gamma treated PIEC did so, the peak response being at day 5 (Fig. 13).

Interferon gamma treated PIEC clones carrying the empty vector, the pcDNA3mutCIITA2 construct or the pcDNA3mutCIITA3 construct behaved like normal PIEC cells. However, the PIEC clones carrying the pcDNA3mutCIITA4 construct failed completely to stimulate the CD4+ T cells. Thus the suppression of MHC class II expression profoundly inhibited the functional capacity of the PIEC clones to stimulate direct human T cell xenorecognition.

## DISCUSSION

The acute shortage of human organs for transplantation has resulted in intensive efforts to identify additional sources of donor organs. The possibility of using other species, in particular the pig, as a novel source of grafts for man has been perceived in recent years as the only avenue offering substantive hope of alleviating the donor shortage. Intensive research efforts have brought the field to a stage where clinical trials of porcine organ xenografts to man might soon begin<sup>22</sup>.

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5 The major immediate problem with porcine organ xenografts to man and to other old world primates has been natural antibody- and complement-mediated hyperacute rejection, which destroys organ xenografts within a few minutes or hours of transplantation. This potent and seemingly insuperable barrier appears now to have been overcome by the use of transgenic pig donors expressing human complement regulators<sup>23</sup>.

15 Now that one can look beyond hyperacute rejection, it is clear that there are important barriers still to be overcome. Some of these barriers are likely to encompass rejection mechanisms not seen with allografts, in particular involving NK cells, macrophages and granulocytes<sup>24-26</sup>. However, one of the important barriers will be that of T cell-mediated rejection, involving both direct T cell recognition of SLA-DR and SLA-DQ antigens, and indirect T cell recognition of the many thousands of foreign proteins present in porcine organs<sup>1,27</sup>.

25 Our studies are aimed at controlling the human anti pig direct T cell xenorecognition response, and the various immune effector mechanisms mediated or facilitated by this response<sup>1</sup>. In transplantation, two donor cell types are likely to be the major stimulants of direct T cell recognition: the migratory leucocytes known as interstitial dendritic cells<sup>28</sup> and MHC class II positive VEC<sup>29,30</sup>.

30 Whereas donor interstitial dendritic cells are transient

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5 components of the graft, emigrating within 1 or 2 weeks of  
transplantation<sup>31</sup>, VECs are present for the life-time of the  
graft.

10 It has been proposed that the MHC class II positive VECs of  
allografted organs represent a strong, constant stimulus for  
direct T cell allorecognition in clinical transplantation,  
and that the suppression of MHC class II expression on VECs  
in allografted organs might markedly reduce the strength of  
rejection response, in particular chronic rejection  
responses<sup>32</sup>. Direct T cell xenorecognition of donor VEC  
15 therefore is likely to be of particular importance in the  
transplantation of porcine organ xenografts to man. This  
species difference is almost certainly the basis for the  
higher precursor frequency of human CD4+ T cells for direct  
xenorecognition of porcine VECs as compared to direct  
20 allorecognition of human VECs<sup>4</sup>.

The longer term survival of transgenic porcine organ  
xenografts in old world monkeys requires high levels of  
immunosuppression<sup>33</sup>. The suppression of SLA-DR and SLA-DQ  
25 expression on the VECs of porcine organ xenografts with the  
mutant human CIITA constructs of the present invention  
enable the reduction and even complete abolition of the  
direct T cell recognition response in human recipients of  
porcine xenografts, This will facilitate the long-term  
30 acceptance of porcine xenografts with clinically acceptable

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levels of immunosuppression.

5

It is important to note that total suppression of MHC class II expression on APCs is not required for substantial reduction in their antigen-presenting capacity. For example, Hatano et al<sup>34</sup> were able to abolish the capacity of  
10 B cells to present antigen to T cell clones by suppressing MHC class II expression by only ~80%, using synthetic antisense oligonucleotides to MHC class II mRNA. In our work, the residual low levels (~5%) of SLA-DR and SLA-DQ in porcine PIEC were unable to stimulate direct xenorecognition  
15 by human T cells.

The inability of pcDNA3mutCIITA3 to suppress porcine MHC class II expression was unexpected, in view of its established ability to suppress human MHC class II  
20 expression<sup>14</sup>, and the effectiveness of pcDNA3mutCIITA4. The absence of mutCIITA3 mRNA in the pcDNA3mutCIITA3 transfected cells indicates that the problem with this construct might rest with the stability of the mRNA in porcine cells, and not with any specific requirement in the pig for the NLS of  
25 the large T antigens of SV40, which is present in mutCIITA4.

CIITA has recently been shown to play a role in upregulating the expression of HLA class I heavy chain genes, but not the other genes involved in the cell surface expression of MHC  
30 class I molecules (e.g.  $\beta$ 2 microglobulin, TAP, LMP)<sup>35,36</sup>.

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However, in our studies, suppression of CIITA did not influence porcine MHC class I expression. In addition, 5 although CIITA independent expression of HLA-DQ has been demonstrated in EBV transformed B cells<sup>13</sup>, we did not see a differential effect on SLA-DR or SLA-DQ in our studies.

Double transgenic pigs, involving a human regulator of 10 complement (to prevent hyperacute rejection) and a mutated human CIITA construct of the present invention preferably under the control of a constitutive VEC specific promoter (to suppress direct T cell xenorecognition), will provide an important advance in the development of porcine xenografts 15 for clinical transplantation.



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SEQ ID NO: 1:

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TGCCACCATG	GAGTTGGGGC	CCCTAGAAGG	TGGCTACCTG	GAGCTTCTTA	ACAGCGATGC	240
TGACCCCCTG	TGCCTCTACC	ACTTCTATGA	CCAGATGGAC	CTGGCTGGAG	AAGAAGAGAT	300
TGAGCTCTAC	TCAGAACCCG	ACACAGACAC	CATCAACTGC	GACCAGTTCA	GCAGGCTGTT	360
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ACTTCTTCTC	AGTCACAGCC	ACAGCCCTAC	TTTGTGCCGG	GCAGTGTGCC	AGCTCTCAGA	1980
GGCCCTGCTG	GAGCTTGGGG	AGGACGCCAA	GCTGCCCTCC	ACGCTCACGG	GACTCTATGT	2040
CGGCCTGCTG	GGCCGTGCAG	CCCTCGACAG	CCCCCCCCGG	GCCCTGGCAG	AGCTGGCCAA	2100
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GAGGACACTA	ACCACGCTGG	ACCTTGAAC	GGTACTTGT	GGACACAGCT	CTTCTCCAGG	3600
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CGAGGCCTTC	TCTGAAGGAC	ATTGCGGACA	GCCACGGCCA	GGCCAGAGGG	AGTGACAGAG	3840
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SEQ ID NO: 2:

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His	Gly	Glu	Val	Pro	Gln	Ala	Ser	Gln	Val	Pro	Pro	Pro	Ser	Gly	Phe	260	265	270	

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Cys	Pro	Ala	Ala	Gly	Glu	Val	Ser	Asn	Lys	Leu	Pro	Lys	Trp	Pro	Glu
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Asp Ala Leu Phe Glu Leu Ser Gly Phe Ser Met Glu Gln Ala Gln Ala  
 565 570 575  
 Tyr Val Met Arg Tyr Phe Glu Ser Ser Gly Met Thr Glu His Gln Asp  
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 595 600 605  
 His Ser Pro Thr Leu Cys Arg Ala Val Cys Gln Leu Ser Glu Ala Leu  
 610 615 620  
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 625 630 635 640  
 Tyr Val Gly Leu Leu Gly Arg Ala Ala Leu Asp Ser Pro Pro Gly Ala  
 645 650 655  
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 Ser Thr Leu Gln Glu Asp Gln Phe Pro Ser Ala Asp Val Arg Thr Trp  
 675 680 685  
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 690 695 700  
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 725 730 735  
 Leu Ala Leu Thr Pro Arg Lys Lys Arg Pro Tyr Asp Asn Trp Leu Glu  
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 Lys Gln Lys Val Leu Ala Arg Tyr Leu Lys Arg Leu Gln Pro Gly Thr  
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 835 840 845

- 65 -

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 Ala Gln Gln Leu Ala Ala Ser Leu Arg Arg Cys Pro His Val Glu Thr  
 1090 1095 1100  
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 Gln Gln Gln Asp Ser Arg Ile Ser Leu Arg  
 1125 1130

SEQ.ID.NO:3

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GGCTGCCAAG GAGCACCGGC GGCCGCGTGA GACACGAGTG ATTGCTGTGC TGGGCAAAGC  
TGGTCAGGGC AAGAGCTATT GGGCTGGGGC AGTGAGCCGG GCCTGGGCTT GTGGCCGGCT  
TCCCCAGTAC GACTTTGTCT TCTCTGTCCC CTGCCATTGC TTGAACCGTC CGGGGGATGC  
CTATGGCCTG CAGGATCTGC TCTTCTCCCT GGGCCACAG CCACTCGTGG CGGCCGATGA  
GGTTTTCAGC CACATCTTGA AGAGACCTGA CCGCGTTCTG CTCATCCTAG ACGCCTTCGA



GGAGCTGGAA GCGCAAGATG GCTTCCTGCA CAGCACGTGC GGACCGGCAC CGGCGGAGCC  
CTGCTCCCTC CGGGGGCTGC TGGCCGGCCT TTTCCAGAAG AAGCTGCTCC GAGGTTGCAC  
CCTCCTCCTC ACAGCCCGGC CCCGGGGCCG CCTGGTCCAG AGCCTGAGCA AGGCCGACGC  
CCTATTTGAG CTGTCCGGCT TCTCCATGGA GCAGGCCAG GCATACGTGA TGCCTACTT  
TGAGAGCTCA GGGATGACAG AGCACCAAGA CAGAGCCCTG ACGCTCCTCC GGGACCGGCC  
ACTTCTTCTC AGTCACAGCC ACAGCCCTAC TTTGTGCCGG GCAGTGTGCC AGCTCTCAGA  
GGCCCTGCTG GAGCTTGGGG AGGACGCCAA GCTGCCCTCC ACGCTCACGG GACTCTATGT  
CGGCCTGCTG GGCCGTGCAG CCCTCGACAG CCCCCCGGG GCCCTGGCAG AGCTGGCCAA  
GCTGGCCTGG GAGCTGGGCC GCAGACATCA AAGTACCCTA CAGGAGGACC AGTTCCCATC  
CGCAGACGTG AGGACCTGGG CGATGGCCAA AGGCTTAGTC CAACACCCAC CGCGGGCCGC  
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CCTCCGCAGC ACTGGCATTT GCCCCTCTGG ATTGGGGAGC CTCGTGGGAC TCAGCTGTGT  
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CCTGGCCAGA TGCACCAGGC CCTTAGCAGG GAAACAGCTA ATGGGACACT AATGGGGCGG  
TGAGAGGGGA ACAGACTGGA AGCACAGCTT CATTTCTCTGT GTCTTTTTTTC ACTACATTAT  
AAATGTCTCT TTAATGTCAC AAAAAAAAAA AAAAAAAAAA AAA

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SEQ ID NO: 4:

Note: Numbering is as in the native human CIITA, see  
SEQ.ID.NO:2

																	Met
																	1
Arg	Cys	Leu	Ala	Pro	Ile	Ile	Pro	Ala	Asp	Leu	Lys	His	Trp	Lys	Pro		
		5			150					155					160		
Ala	Glu	Pro	Pro	Thr	Val	Val	Thr	Gly	Ser	Leu	Leu	Val	Gly	Pro	Val		
				165					170					175			
Ser	Asp	Cys	Ser	Thr	Leu	Pro	Cys	Leu	Pro	Leu	Pro	Ala	Leu	Phe	Asn		
			180					185					190				
Gln	Glu	Pro	Ala	Ser	Gly	Gln	Met	Arg	Leu	Glu	Lys	Thr	Asp	Gln	Ile		
		195					200					205					
Pro	Met	Pro	Phe	Ser	Ser	Ser	Ser	Leu	Ser	Cys	Leu	Asn	Leu	Pro	Glu		
	210					215					220						
Gly	Pro	Ile	Gln	Phe	Val	Pro	Thr	Ile	Ser	Thr	Leu	Pro	His	Gly	Leu		
225					230					235					240		
Trp	Gln	Ile	Ser	Glu	Ala	Gly	Thr	Gly	Val	Ser	Ser	Ile	Phe	Ile	Tyr		
				245					250					255			
His	Gly	Glu	Val	Pro	Gln	Ala	Ser	Gln	Val	Pro	Pro	Pro	Ser	Gly	Phe		
			260					265					270				
Thr	Val	His	Gly	Leu	Pro	Thr	Ser	Pro	Asp	Arg	Pro	Gly	Ser	Thr	Ser		
		275					280					285					
Pro	Phe	Ala	Pro	Ser	Ala	Thr	Asp	Leu	Pro	Ser	Met	Pro	Glu	Pro	Ala		
	290					295					300						
Leu	Thr	Ser	Arg	Ala	Asn	Met	Thr	Glu	His	Lys	Thr	Ser	Pro	Thr	Gln		
305					310					315					320		

Cys	Pro	Ala	Ala	Gly	Glu	Val	Ser	Asn	Lys	Leu	Pro	Lys	Trp	Pro	Glu	
				325					330					335		
Pro	Val	Glu	Gln	Phe	Tyr	Arg	Ser	Leu	Gln	Asp	Thr	Tyr	Gly	Ala	Glu	
			340					345					350			
Pro	Ala	Gly	Pro	Asp	Gly	Ile	Leu	Val	Glu	Val	Asp	Leu	Val	Gln	Ala	
		355					360					365				
Arg	Leu	Glu	Arg	Ser	Ser	Ser	Lys	Ser	Leu	Glu	Arg	Glu	Leu	Ala	Thr	
	370					375					380					
Pro	Asp	Trp	Ala	Glu	Arg	Gln	Leu	Ala	Gln	Gly	Gly	Leu	Ala	Glu	Val	
385					390					395					400	
Leu	Leu	Ala	Ala	Lys	Glu	His	Arg	Arg	Pro	Arg	Glu	Thr	Arg	Val	Ile	
				405					410					415		
Ala	Val	Leu	Gly	Lys	Ala	Gly	Gln	Gly	Lys	Ser	Tyr	Trp	Ala	Gly	Ala	
			420					425					430			
Val	Ser	Arg	Ala	Trp	Ala	Cys	Gly	Arg	Leu	Pro	Gln	Tyr	Asp	Phe	Val	
		435					440					445				
Phe	Ser	Val	Pro	Cys	His	Cys	Leu	Asn	Arg	Pro	Gly	Asp	Ala	Tyr	Gly	
	450					455					460					
Leu	Gln	Asp	Leu	Leu	Phe	Ser	Leu	Gly	Pro	Gln	Pro	Leu	Val	Ala	Ala	
465					470					475					480	
Asp	Glu	Val	Phe	Ser	His	Ile	Leu	Lys	Arg	Pro	Asp	Arg	Val	Leu	Leu	
				485					490					495		
Ile	Leu	Asp	Ala	Phe	Glu	Glu	Leu	Glu	Ala	Gln	Asp	Gly	Phe	Leu	His	
			500					505					510			
Ser	Thr	Cys	Gly	Pro	Ala	Pro	Ala	Glu	Pro	Cys	Ser	Leu	Arg	Gly	Leu	
		515					520					525				
Leu	Ala	Gly	Leu	Phe	Gln	Lys	Lys	Leu	Leu	Arg	Gly	Cys	Thr	Leu	Leu	
	530					535					540					
Leu	Thr	Ala	Arg	Pro	Arg	Gly	Arg	Leu	Val	Gln	Ser	Leu	Ser	Lys	Ala	
545					550					555					560	
Asp	Ala	Leu	Phe	Glu	Leu	Ser	Gly	Phe	Ser	Met	Glu	Gln	Ala	Gln	Ala	
				565					570					575		
Tyr	Val	Met	Arg	Tyr	Phe	Glu	Ser	Ser	Gly	Met	Thr	Glu	His	Gln	Asp	
			580					585					590			
Arg	Ala	Leu	Thr	Leu	Leu	Arg	Asp	Arg	Pro	Leu	Leu	Leu	Ser	His	Ser	
		595					600					605				

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His	Ser	Pro	Thr	Leu	Cys	Arg	Ala	Val	Cys	Gln	Leu	Ser	Glu	Ala	Leu
	610					615					620				
Leu	Glu	Leu	Gly	Glu	Asp	Ala	Lys	Leu	Pro	Ser	Thr	Leu	Thr	Gly	Leu
625					630					635					640
Tyr	Val	Gly	Leu	Leu	Gly	Arg	Ala	Ala	Leu	Asp	Ser	Pro	Pro	Gly	Ala
				645					650					655	
Leu	Ala	Glu	Leu	Ala	Lys	Leu	Ala	Trp	Glu	Leu	Gly	Arg	Arg	His	Gln
			660					665					670		
Ser	Thr	Leu	Gln	Glu	Asp	Gln	Phe	Pro	Ser	Ala	Asp	Val	Arg	Thr	Trp
		675					680					685			
Ala	Met	Ala	Lys	Gly	Leu	Val	Gln	His	Pro	Pro	Arg	Ala	Ala	Glu	Ser
	690					695					700				
Glu	Leu	Ala	Phe	Pro	Ser	Phe	Leu	Leu	Gln	Cys	Phe	Leu	Gly	Ala	Leu
705					710					715					720
Trp	Leu	Ala	Leu	Ser	Gly	Glu	Ile	Lys	Asp	Lys	Glu	Leu	Pro	Gln	Tyr
				725					730					735	
Leu	Ala	Leu	Thr	Pro	Arg	Lys	Lys	Arg	Pro	Tyr	Asp	Asn	Trp	Leu	Glu
			740					745					750		
Gly	Val	Pro	Arg	Phe	Leu	Ala	Gly	Leu	Ile	Phe	Gln	Pro	Pro	Ala	Arg
		755					760					765			
Cys	Leu	Gly	Ala	Leu	Leu	Gly	Pro	Ser	Ala	Ala	Ala	Ser	Val	Asp	Arg
	770					775						780			
Lys	Gln	Lys	Val	Leu	Ala	Arg	Tyr	Leu	Lys	Arg	Leu	Gln	Pro	Gly	Thr
785				790						795					800
Leu	Arg	Ala	Arg	Gln	Leu	Leu	Glu	Leu	Leu	His	Cys	Ala	His	Glu	Ala
				805					810					815	
Glu	Glu	Ala	Gly	Ile	Trp	Gln	His	Val	Val	Gln	Glu	Leu	Pro	Gly	Arg
			820					825					830		
Leu	Ser	Phe	Leu	Gly	Thr	Arg	Leu	Thr	Pro	Pro	Asp	Ala	His	Val	Leu
		835					840					845			
Gly	Lys	Ala	Leu	Glu	Ala	Ala	Gly	Gln	Asp	Phe	Ser	Leu	Asp	Leu	Arg
	850					855					860				
Ser	Thr	Gly	Ile	Cys	Pro	Ser	Gly	Leu	Gly	Ser	Leu	Val	Gly	Leu	Ser
865					870					875					880
Cys	Val	Thr	Arg	Phe	Arg	Ala	Ala	Leu	Ser	Asp	Thr	Val	Ala	Leu	Trp
				885					890					895	

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Glu	Ser	Leu	Arg	Gln	His	Gly	Glu	Thr	Lys	Leu	Leu	Gln	Ala	Ala	Glu	
			900					905					910			
Glu	Lys	Phe	Thr	Ile	Glu	Pro	Phe	Lys	Ala	Lys	Ser	Leu	Lys	Asp	Val	
		915					920					925				
Glu	Asp	Leu	Gly	Lys	Leu	Val	Gln	Thr	Gln	Arg	Thr	Arg	Ser	Ser	Ser	
	930					935					940					
Glu	Asp	Thr	Ala	Gly	Glu	Leu	Pro	Ala	Val	Arg	Asp	Leu	Lys	Lys	Leu	
945					950					955						960
Glu	Phe	Ala	Leu	Gly	Pro	Val	Ser	Gly	Pro	Gln	Ala	Phe	Pro	Lys	Leu	
				965					970					975		
Val	Arg	Ile	Leu	Thr	Ala	Phe	Ser	Ser	Leu	Gln	His	Leu	Asp	Leu	Asp	
			980					985					990			
Ala	Leu	Ser	Glu	Asn	Lys	Ile	Gly	Asp	Glu	Gly	Val	Ser	Gln	Leu	Ser	
		995					1000					1005				
Ala	Thr	Phe	Pro	Gln	Leu	Lys	Ser	Leu	Glu	Thr	Leu	Asn	Leu	Ser	Gln	
	1010					1015					1020					
Asn	Asn	Ile	Thr	Asp	Leu	Gly	Ala	Tyr	Lys	Leu	Ala	Glu	Ala	Leu	Pro	
1025					1030					1035					1040	
Ser	Leu	Ala	Ala	Ser	Leu	Leu	Arg	Leu	Ser	Leu	Tyr	Asn	Asn	Cys	Ile	
				1045					1050					1055		
Cys	Asp	Val	Gly	Ala	Glu	Ser	Leu	Ala	Arg	Val	Leu	Pro	Asp	Met	Val	
			1060					1065					1070			
Ser	Leu	Arg	Val	Met	Asp	Val	Gln	Tyr	Asn	Lys	Phe	Thr	Ala	Ala	Gly	
		1075					1080					1085				
Ala	Gln	Gln	Leu	Ala	Ala	Ser	Leu	Arg	Arg	Cys	Pro	His	Val	Glu	Thr	
	1090					1095					1100					
Leu	Ala	Met	Trp	Thr	Pro	Thr	Ile	Pro	Phe	Ser	Val	Gln	Glu	His	Leu	
1105					1110					1115					1120	
Gln	Gln	Gln	Asp	Ser	Arg	Ile	Ser	Leu	Arg							
				1125					1130							

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## CLAIMS:

1. A polypeptide that comprises the amino acid sequence of a class II trans activator (CIITA) protein from the N-terminus of which amino acid residues are missing such that the resulting polypeptide reduces the expression of MHC class II antigens.
2. A polypeptide as claimed in claim 1, wherein all of the acidic amino terminal transcriptional activation domain is missing, or a part of the acidic amino terminal transcriptional activation domain sufficient to suppress transcriptional activation activity is missing.
3. A polypeptide as claimed in claim 2, wherein the acidic amino terminal transcriptional activation domain comprises or consists of at least amino acids 1 to 114 of human CIITA or the corresponding amino acids of a CIITA of another species.
4. A polypeptide as claimed in claim 3, wherein the acidic amino terminal transcriptional activation domain comprises or consists of at least amino acids 1 to 125 of human CIITA or the corresponding amino acids of a CIITA of another species.
5. A polypeptide as claimed in any one of claims 1 to 4, wherein at least part of the carboxy terminal regions rich in proline, serine and threonine are present.
6. A polypeptide as claimed in claim 5, wherein all of the carboxy terminal regions rich in proline, serine and threonine are present.
7. A polypeptide as claimed in claim 5 or claim 6, wherein the carboxy terminal regions rich in proline, serine and threonine extend from about amino acid 163 or 166 in the human CIITA or from the corresponding position in a CIITA of another species towards the C-terminus.

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8. A polypeptide as claimed in any one of claims 1 to 7, wherein the N-terminus of the polypeptide starts at amino acid 114 or 115 human CIITA or at the corresponding position in a CIITA of another species, or at a position downstream thereof, for example, at position 125 or 126 or downstream thereof, for example, at position 135 or 136 or downstream thereof, for example, at position 140 or 141 or downstream thereof, for example, at position 145 or 146.

9. A polypeptide as claimed in any one of claims 1 to 8, wherein the N-terminus of the polypeptide is at amino acid 166, 165, 164, 163 or 162 of human CIITA, or the corresponding position in a CIITA of another species, or at a position upstream thereof, for example, at position 161 or 160 or upstream thereof, for example, at position 156 or 155 or upstream thereof, for example, at position 152, 151 or 150.

10. A polypeptide as claimed in any one of claims 1 to 8, wherein the N-terminus of the polypeptide starts at an amino acid at any of positions 140 to 156 of human CIITA or at the corresponding position in a CIITA of another species.

11. A polypeptide as claimed in any one of claims 1 to 8, wherein the N-terminus of the polypeptide starts at an amino acid at any of positions 145 to 152 of human CIITA or at the corresponding position in a CIITA of another species.

12. A polypeptide as claimed in any one of claims 1 to 11, wherein the CIITA protein is a human CIITA protein.

13. A polypeptide as claimed in any one of claims 1 to 11, wherein the CIITA protein is a porcine CIITA protein.

14. A polypeptide as claimed in claim 12, wherein the amino acid sequence of the CIITA protein is as set out in Figure 5 (SEQ.ID.NO.1) herein.

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15. A polypeptide as claimed in claim 1, having the amino acid sequence set out in Figure 14 (SEQ.ID.NO.2) herein.

16. A ribozyme that is targeted at bases 1159-1161 (GUA) of human CIITA mRNA or at the corresponding target in CIITA mRNA of another species.

17. A nucleic acid molecule that encodes a polypeptide as claimed in any one of claims 1 to 15 or a ribozyme as claimed in claim 16.

18. A nucleic acid molecule as claimed in claim 17, being genomic DNA, cDNA, mRNA or a molecule having the same sequence as cDNA,, mRNA or genomic DNA.

19. A nucleic acid molecule as claimed in claim 18, derived from a sequence as set out in Figure 5 (SEQ.ID.NO.1) herein.

20. A nucleic acid that hybridises selectively to a nucleic acid as claimed in claim 19 and that encodes a polypeptide as claimed in claim 1.

21. A nucleic acid molecule having the sequence set out in Figure 14 (SEQ.ID.NO.2) herein.

22. A construct that comprises a nucleic acid as claimed in any one of claims 17 to 21, in a form suitable for incorporation in a vector or suitable for direct insertion into a host cell.

23. A vector, especially an expression vector, that comprises a nucleic acid as claimed in any one of claims 17 to 21, operably linked to appropriate control sequence(s).

24. A vector as claimed in claim 23, which comprises a tissue-specific promoter, especially a vascular endothelial cell-specific promoter.



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25. A transgenic animal at least some of the cells of which comprise a stably incorporated, functional DNA sequence that encodes a polypeptide as claimed in any one of claims 1 to 15 or that encodes a ribozyme as claimed in claim 16.

26. A transgenic animal as claimed in claim 25, being a transgenic pig.

27. A method for producing a transgenic animal as claimed in claim 25 or claim 26, which comprises stably incorporating the functional DNA sequence that encodes the polypeptide or the ribozyme in one or more cells.

28. A method for producing a transgenic animal comprising microinjection of functional DNA sequence into an embryo, wherein the functional DNA sequence is or comprises a sequence as claimed in any of claims 17 to 21.

29. A cell, tissue or organ that comprises a stably incorporated DNA molecule that encodes a polypeptide as claimed in any one of claims 1 to 15 or that encodes a ribozyme as claimed in claim 16.

30. A transgenic cell as claimed in claim 29, in the form of a cell line that can be maintained in vitro.

31. A polypeptide as claimed in any one of claims 1 to 15 or ribozyme as claimed in claim 16, when obtained by expression in a prokaryotic or eukaryotic host cell in vitro.

32. Use of a nucleic acid molecule as claimed in any one of claims 17 to 21 in the production of a transgenic cell, tissue, organ or animal.

33. A nucleic acid as claimed in any one of claims 17 to 21, for use in the manufacture of a construct or vector for use in the production of a transgenic cell, tissue, organ or animal.

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34. A method of gene therapy for reducing the expression of MHC class II antigens, for example, in the treatment of an autoimmune disease in a human or in a non-human animal, which comprises administering to the human or non-human animal a nucleic acid molecule as claimed in any one of claims 17 to 21, encoding either a mutant CIITA polypeptide as claimed in any one of claims 1 to 15 or a ribozyme as claimed in claim 16.

35. A method as claimed in claim 34, wherein the nucleic acid is administered in a targeted manner such that local immune response suppression is achieved.

36. A method of reducing the expression of MHC class II antigens in a human or non-human animal, which comprises administering to the human or other animal an effective amount of a polypeptide as claimed in any one of claims 1 to 15 or a ribozyme as claimed in claim 16.

37. A method as claimed in claim 36, wherein the human or other animal to be treated has an autoimmune disease or the non-human animal is intended for use as a xenograft donor.

38. A pharmaceutical composition which comprises a nucleic acid molecule as claimed in any one of claims 17 to 21, in a suitable form for use in gene therapy.

39. An antibody to a polypeptide as claimed in any one of claims 1 to 15.

40. A method of animal-to-human transplantation, wherein the transplanted material, for example, cells, tissue or organ, is derived from a transgenic animal as claimed in claim 25 or 26.

41. A pharmaceutical composition which comprises a polypeptide as claimed in any one of claims 1 to 15 or a ribozyme as claimed in claim 16 in admixture or conjunction with a pharmaceutically suitable carrier.

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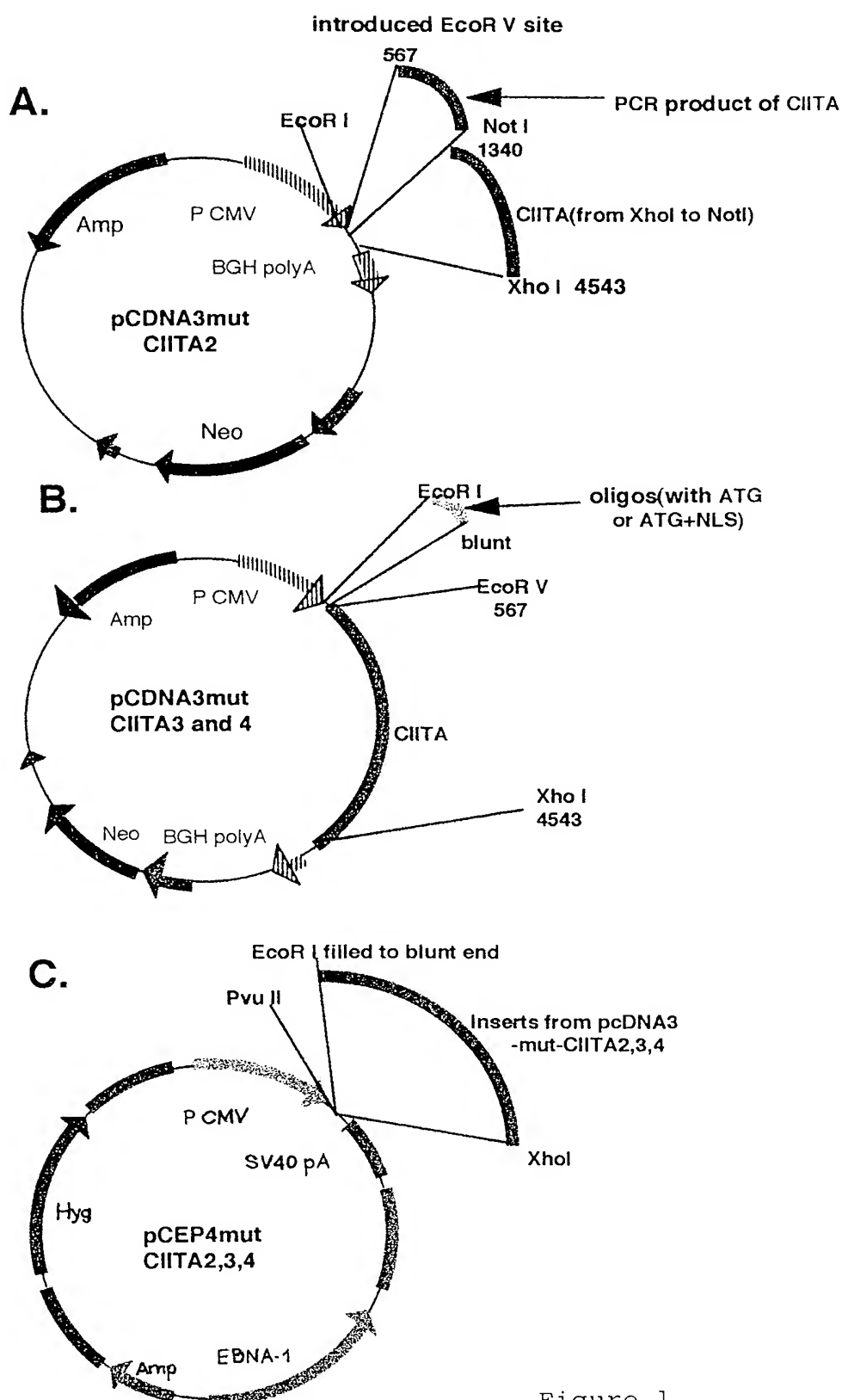


Figure 1

A.



B.

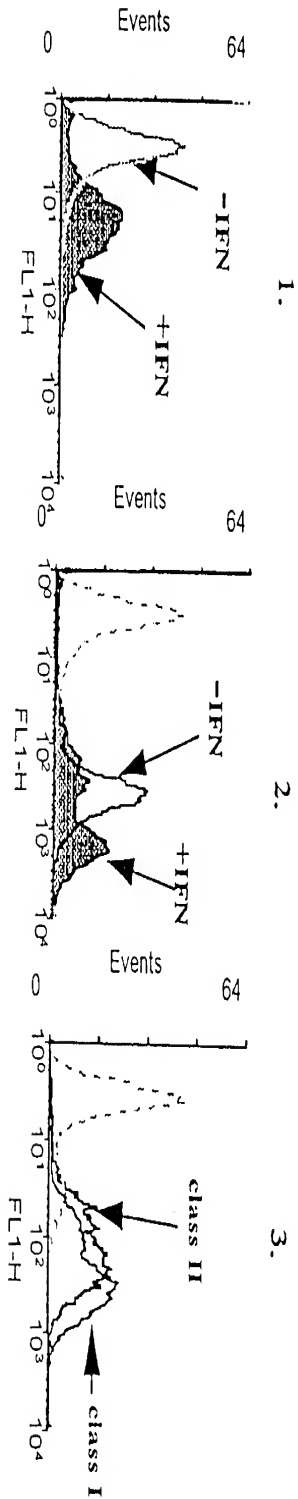


Figure 2

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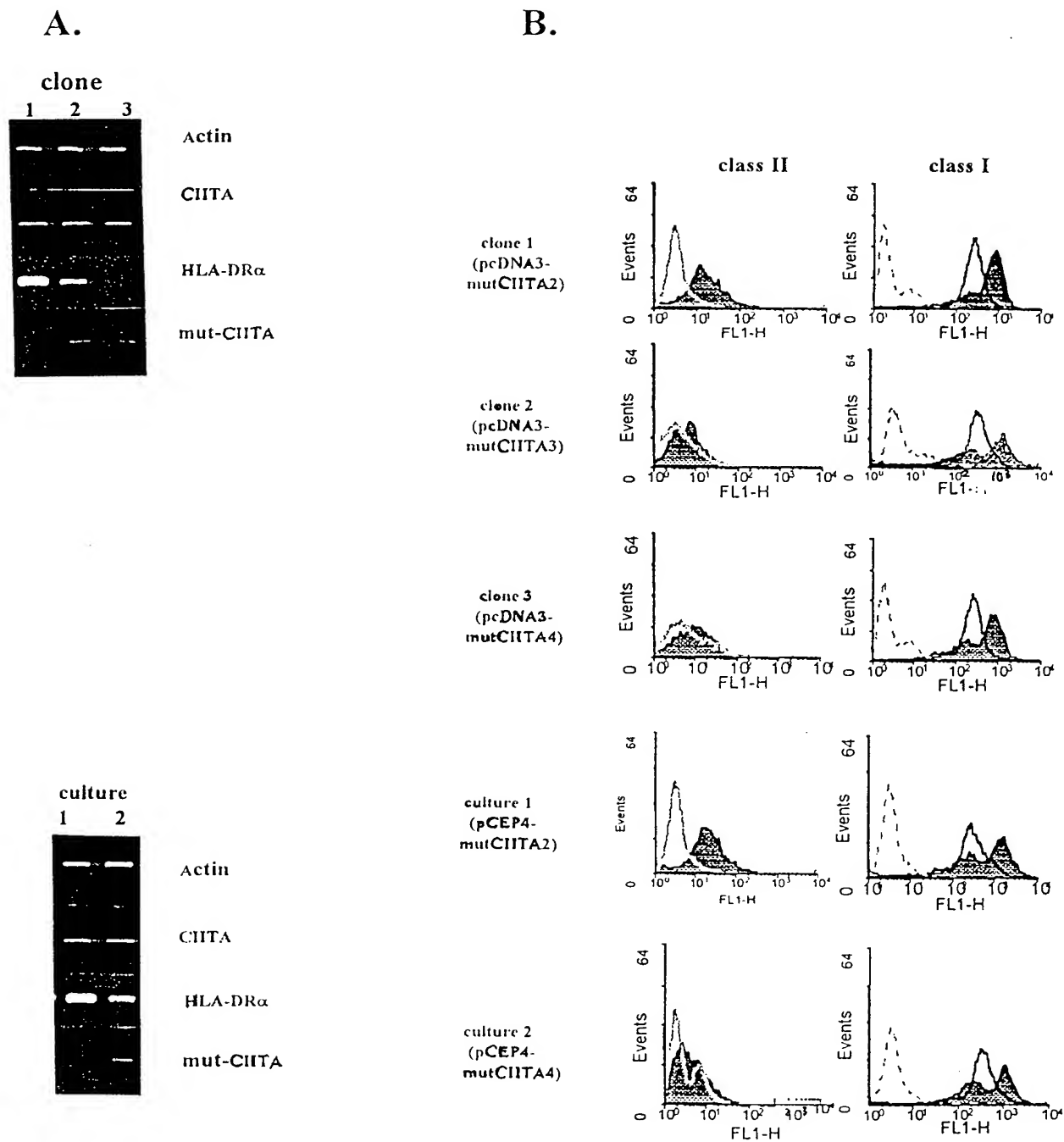


Figure 3

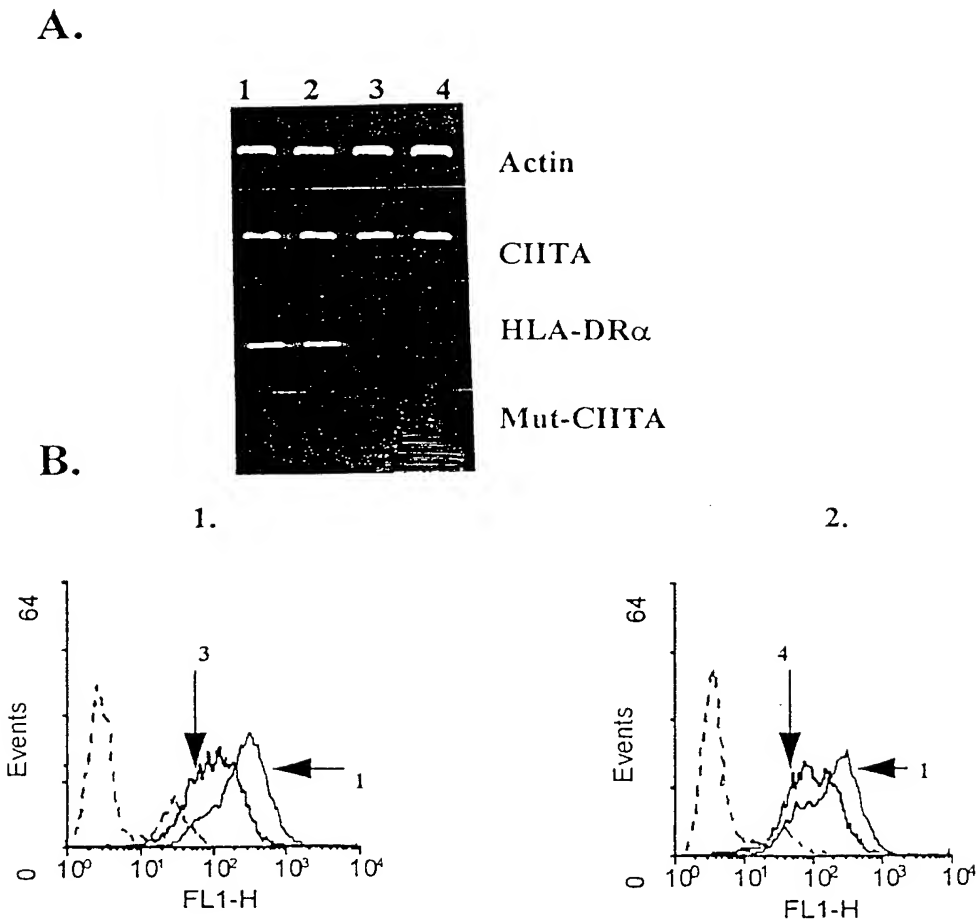


Figure 4

1 1 1  
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 M R C L A P R P A G S Y L S E P Q G S S Q C 22  
 TGCCACCATGGAGTTGGGGCCCTAGAAGGTGGCTACCTGGAGCTTCTTAACAGCGATGCTGACCCCTGTGCCTCTACCAGTTCTATGA 270  
 A T M E L G P L E G G Y L E L L N S D A D P L C L Y H F Y D 52  
 CCAGATCGACCTGGCTGGAGAAGAAGAGATTGAGCTTACTCAGAACCCGACACAGACACCATCAACTGCGACCCAGTTCCAGAGGCTGTT 360  
 Q M D L A G E E E I E L Y S E P D T D T I N C D Q F S R L L 82  
 acidic  
 GTGTGACATCGAAGTGCATGAAGAGACCAGGGAGGCTTATGCCAATATCGCGAAGTGGACCCAGTATGCTTCCAGGACTCCACAGCTGGA 450  
 C D M E G D E E T R E A Y A N I A E L D Q Y V F Q D S Q L E 112  
 GGGCCTGAGCAAGGACATTTTCAAGCACATAGGACCAGATGAAGTGATCGCTGAGAGTATGGAGATGCCAGCACAAGTTGGGCAGAAAAG 540  
 G L S K D I F K H I G P D E V I G E S M E M P A E V G Q K S 142  
 TCAGAAAAGACCCCTTCCCAGAGGAGCTTCCGGCAGACCTGAAGCACTGGAAGCCAGCTGAGCCCCCACTGTGGTGAAGTGGCAGTCTCCT 630  
 Q K R P P P E E L P A D L K H W K P A E P P T V V T G S L L 172  
 I  
 AGTGGACCCAGTGAAGGAGTGTCCACCTGCCCTGCCCTGCCCTGCCCTGTTCAACCAGGAGCCAGGCTCCGGCCAGATGCCGCT 720  
 V G P V S D C S T L P C L P L P A L F N Q E P A S G Q M R L 202  
 II  
 GCAGAAAACCGACAGATTCCCATGCCCTTCTCCAGTTCTCGTTGAGCTGCCCTGAATCTCCCTGAGGGAGCCATCCAGTTTGTCCCCAC 810  
 E K T D Q I P M P F S S S S L S C L N L P E G P I Q F V P T 232

Figure 5/1

CATCTCCACTCTGCCCCATGGGCTCTGCCAAATCTCTGAGGCTGGAACAGGGGTCTCCAGTATATTCATCTACCATGGTGAGGTGCCCCA 900  
 I S T L P H G L W Q I S E A G T G V S S I F I Y H G E V P Q 262

---

III

GGGCAGCCAAGTACCCCTCCAGTGGATTCACTGTCCACGGCTCCCAACATCTCCAGACGGCCAGGCTCCACCAGCCCTTCGCTCC 990  
 A S Q V P P P S G F T V H G L P T S P D R P G S T S P F A P 292

---

ATCAGCCACTGACCTGACCAGCATGCCGCAACCTGCCCTGACCTCCCGAGCAACATGACAGAGCACAAGACGTCCCCCACCCTAATGCC 1080  
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 P A G P D G I L V E V D L V Q A R L E R S S S K S L E R E L 382

GGGCACCCCGGACTGGGCAGAACGGCAGCTGGCCCAAGGAGGCTGGCTGAGGTGCTGTTGGCTGCCAAGGAGCACCGCGGGCGCGCTGA 1350  
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GACACGAGTGATTGCTGTGCTGGGCAAGCTGCTCAGGGCAAGAGCTATTGGCTGGGGCAGTGAGCCGGCCCTGGGCTTGTGGCCGGCT 1440  
 T R V I A V L G K A G Q G K S Y W A G A V S R A W A C G R L 442

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 P Q Y D P V P S V P C H C L N R P G D A Y G L Q D L L P S L 472

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 E L E A Q D G F L H S T C G P A P A E P C S L R G L L A G L 532

Figure 5/2



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F Q K K L L R G C T L L L T A R P R G R L V Q S L S X A D A 592

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L P E L S G P S M E Q A Q A Y V M R Y P E S S G M T E H Q D 622

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R A L T L L R D R P L L L S H S H S P T L C R A V C Q L S E 652

GGCCCTGCTGGAGCTTGGGGAGGACGCCAAGCTGCCCTCCACGCTCAGGGACTCTATGTCCGCCTGCTGGGCGGTGCAGCCCTCGACAG 2070  
A L L E L G E D A K L P S T L T G L Y V G L L G R A A L D S 652

CCCCCCCCGGGGCCCTGGCAGAGCTGCCAAGCTGGCCCTGGGAGCTGGGGCCGAGACATCAAAGTACCCTACAGGAGGACCAGTTCCCATC 2160  
P P G A L A E L A K L A W E L G R R H Q S T L Q E D Q P P S 682

CGCAGACGTGAGGACCTGGCGGATGGCCAAAGCTTAGTCCAACACCCACCGGGGGCCGAGCTCCGAGCTGGCCTTCCCCAGCTTCCT 2250  
A D V R T W A M A K G L V Q H P P R A A E S E L A F P S F L 712

CCTGCAATGCTTCTCGGGGGCCCTGTGGCTGGCTGTGAGTGGCGAAATCAAGGACAAGGAGCTCCCGCAGTACCTAGCATTGACCCCAAG 2340  
L Q C F L G A L W L A L S G E I K D K E L P Q Y L A L T P R 742

GAAGAAGAGGCCCTATGACAACTGGCTGGAGGGCGTCCACGCTTTCTGGCTGGGCTGATCTCCAGCCTCCCGCCCGCTGGCTGGGAGC 2430  
X K R P Y D N W L E G V P R F L A G L I F Q P P A R C L G A 772

CCTACTCGGGCCATCGGGCGCTGCCTCGGTGGACAGGAAGCAGAAGCTGCTTGGAGGTACCTGAAGCGGCTGCAGCCGGGGACACTGCG 2520  
L L G P S A A A S V D R K Q K V L A R Y L K R L Q P G T L R 802

GGCGGGCAGCTGCTTGAGCTGCTGCACTGCGCCACGAGCGCGAGGAGCTGCAATTTGGCAGCACGTGCTACAGGAGCTCCCCGGCGG 2610  
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Figure 5/3

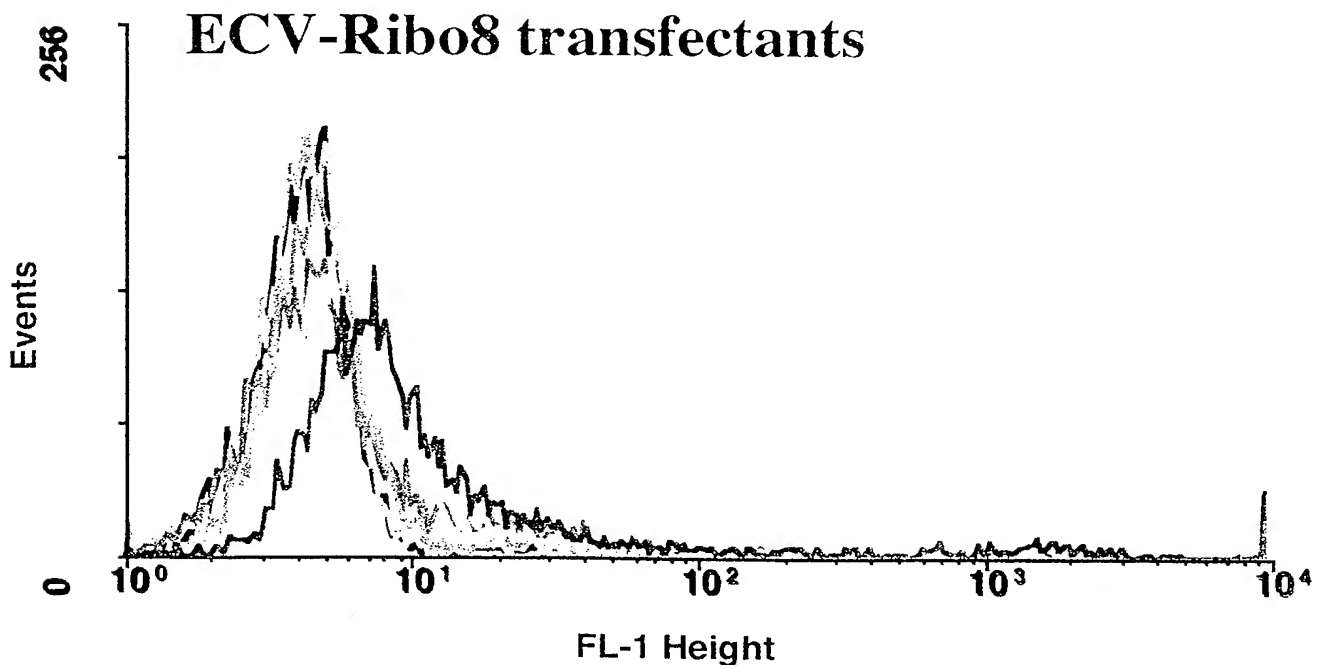
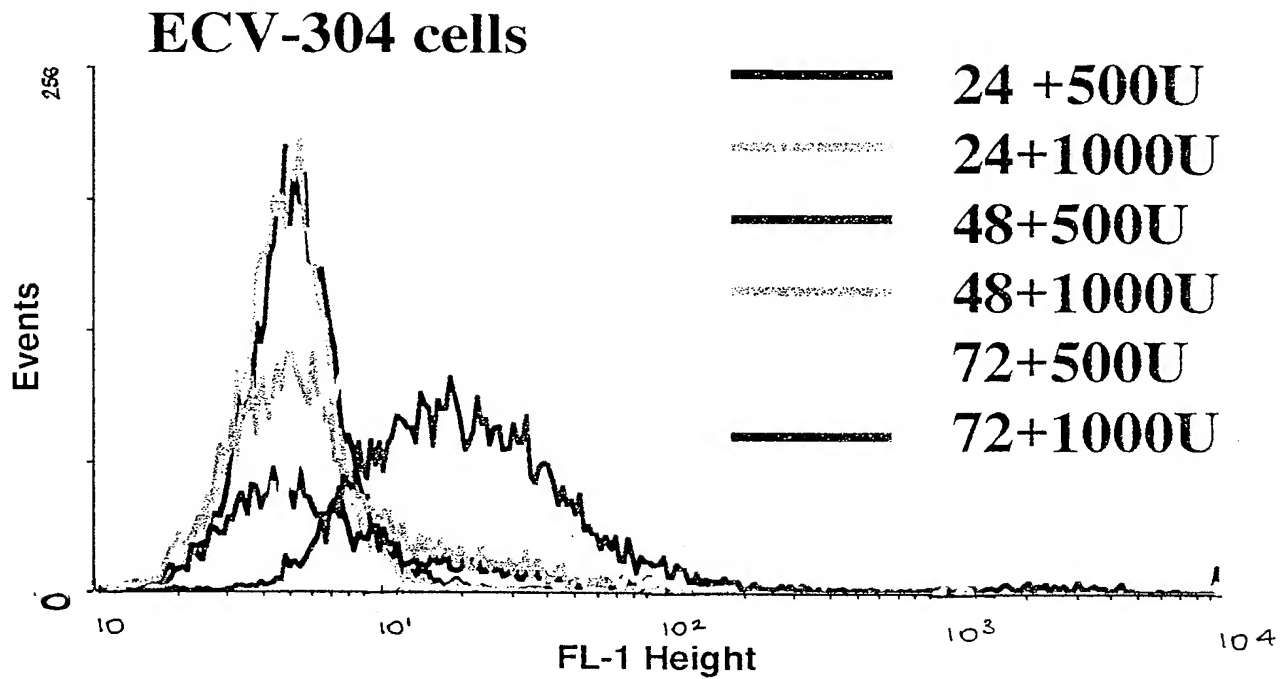
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 CCTCGGAGCACTGGCAATTTGCCCTCTCGATTGGGAGCCCTCGTGGGACTCAGCTGTGTCAACCGTTTTCAGGGCTGCTTCAGCGACAC 2790  
 L R S T G I C P S G L G S L V G L S C V T R F R A A L S D T 892  
  
 CGTGGCGCTGTGGGAGTCCCTGCGGAGCATGCGGAGACCAAGCTACTTCAGGCAAGGAGGAGAGTTTCAACCATCGAGCCCTTTCAAGC 2880  
 V A L M E S L R Q H G E T K L L Q A A E E K F T I E P F K A 922  
  
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 S L R V M D V Q Y N K F T A A G A Q Q L A A S L R R C P H V 1102  
  
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Figure 5/4

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 CTGTATCCCATGAGGCCTCAGCATECTGGCACCCGGCCCTGCTGGTTACGGGTTGCCCCCTGCCCGCTGCCGAATGAACCACATCTTC 3690  
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Figure 5/5

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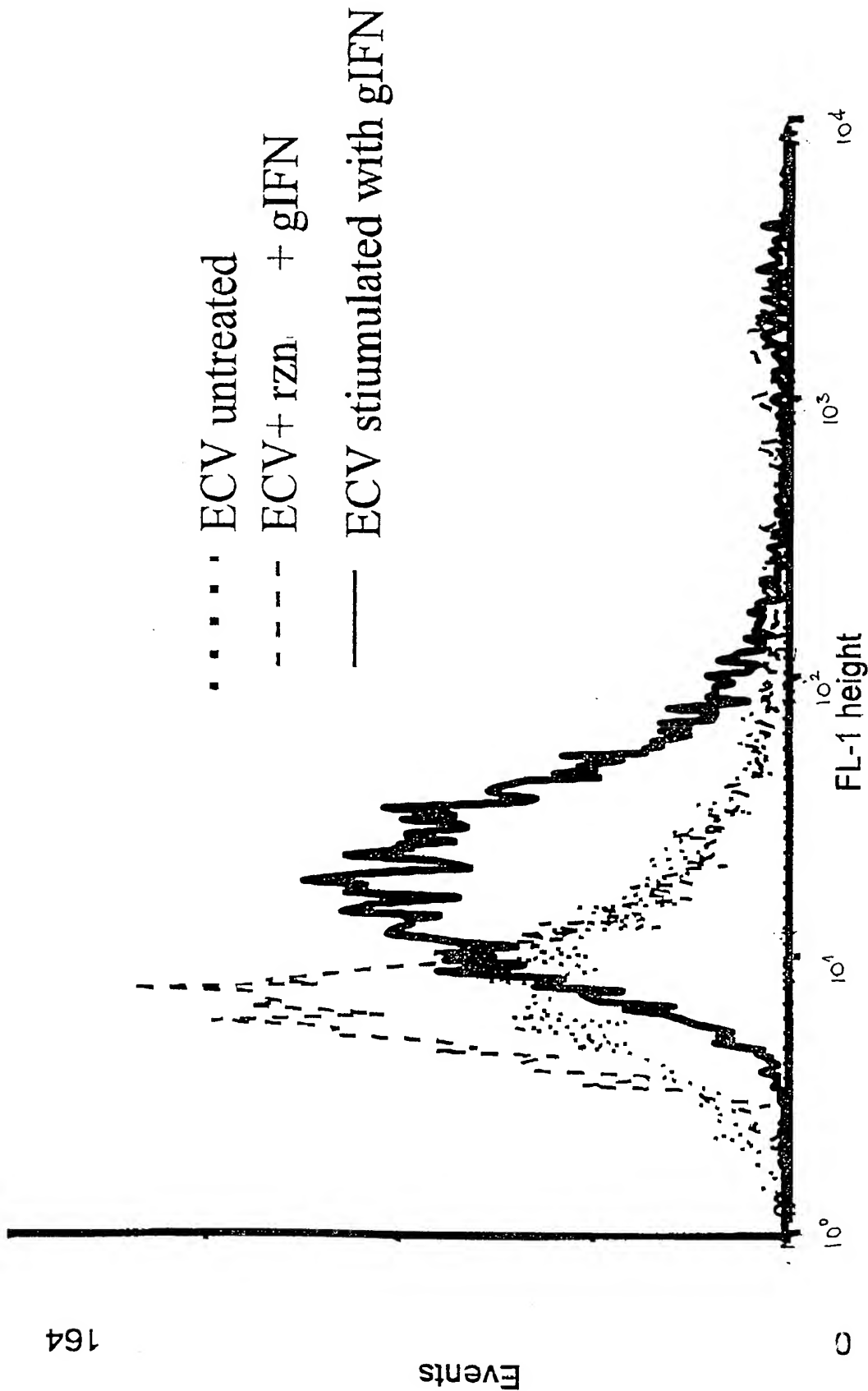


FIGURE 7

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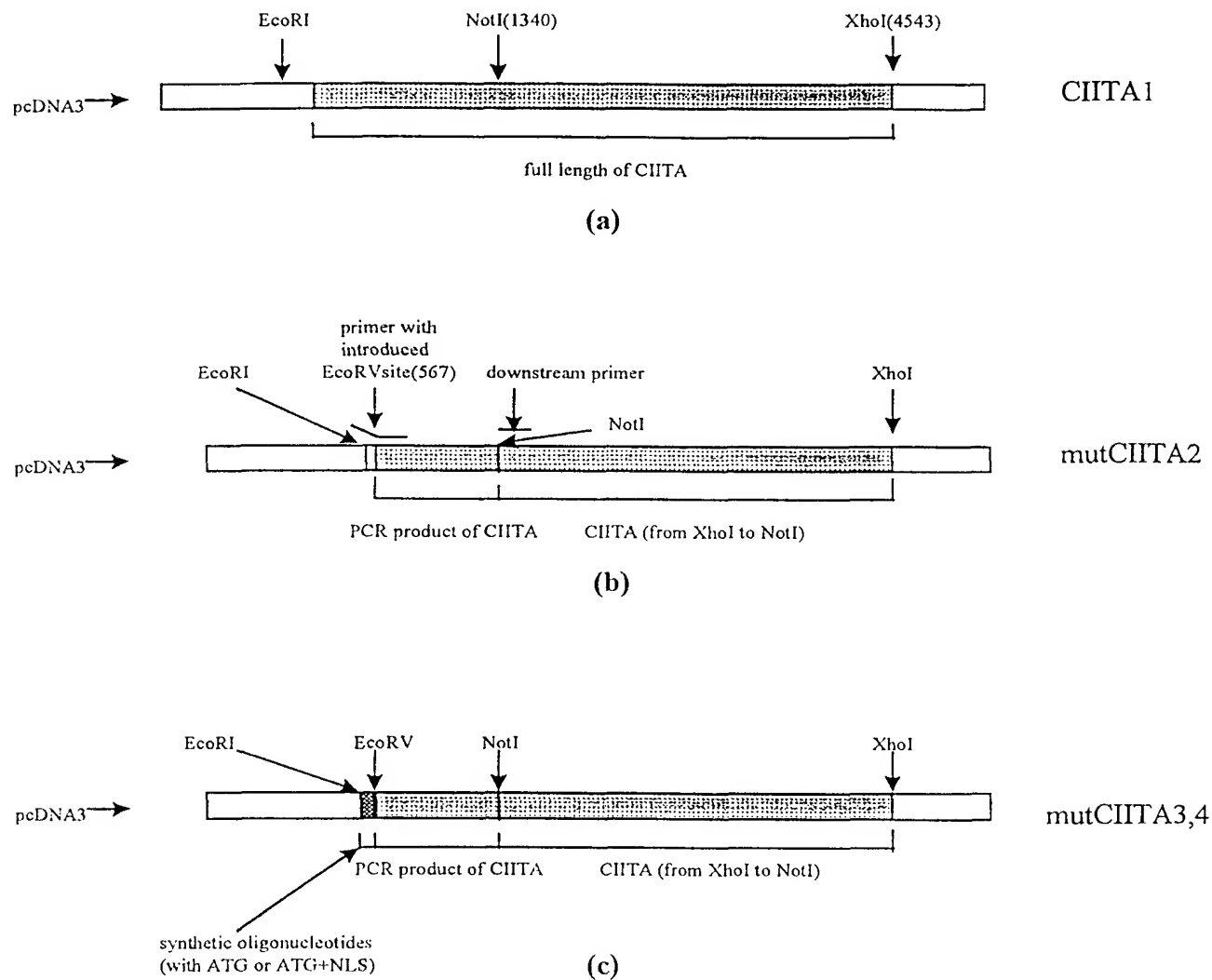


Figure 8

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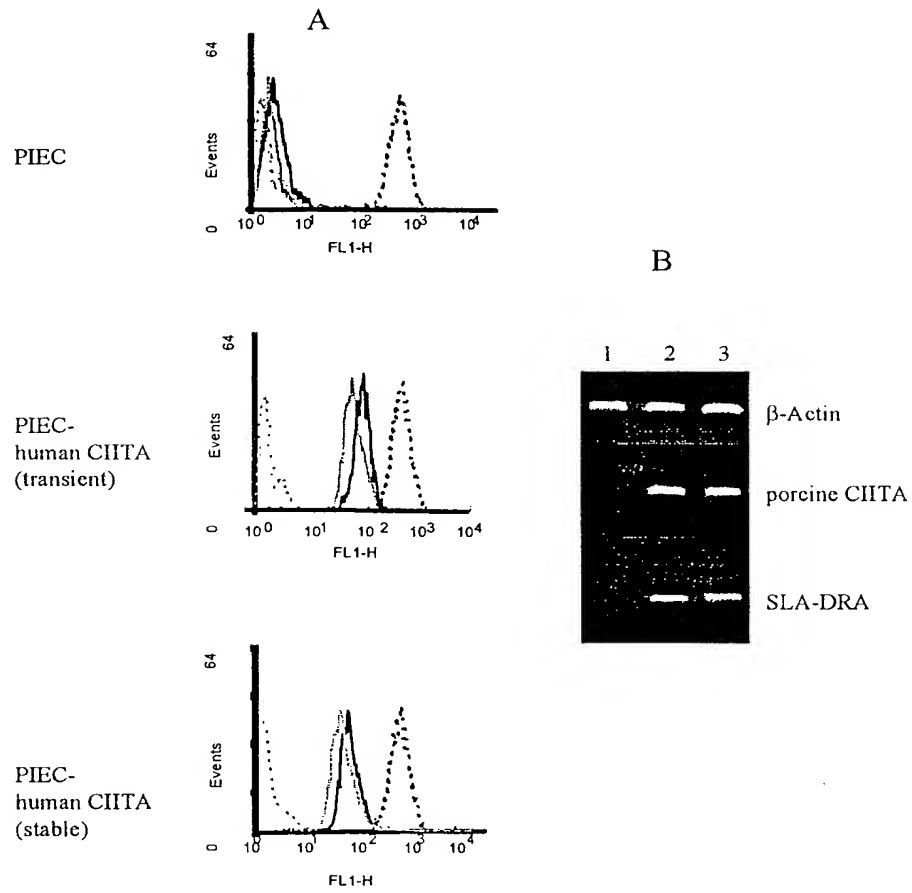


Figure 9

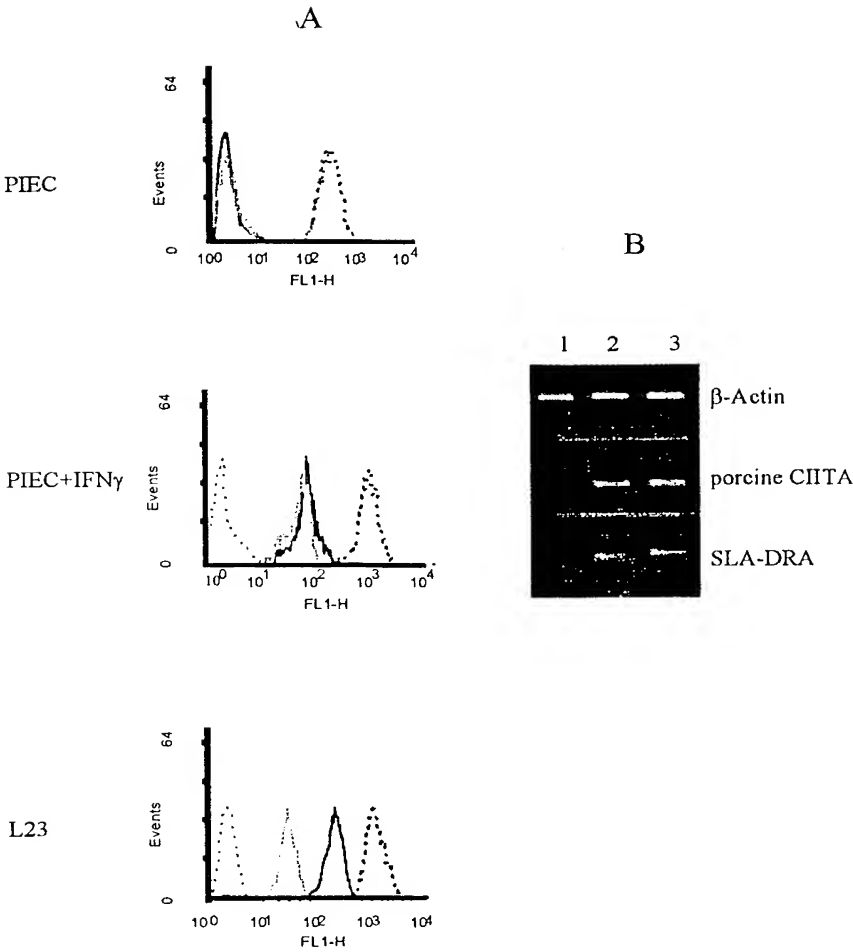


Figure 10



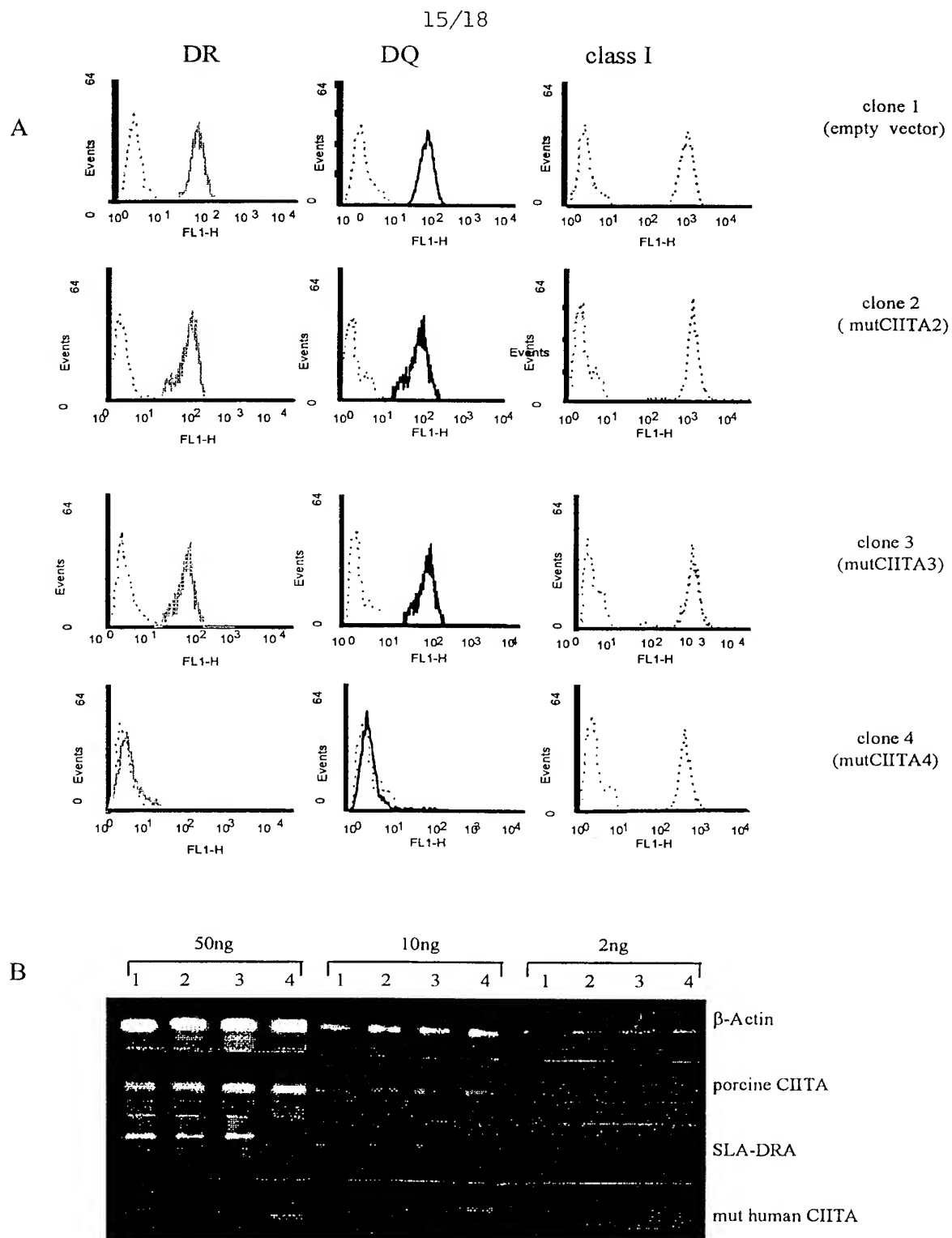
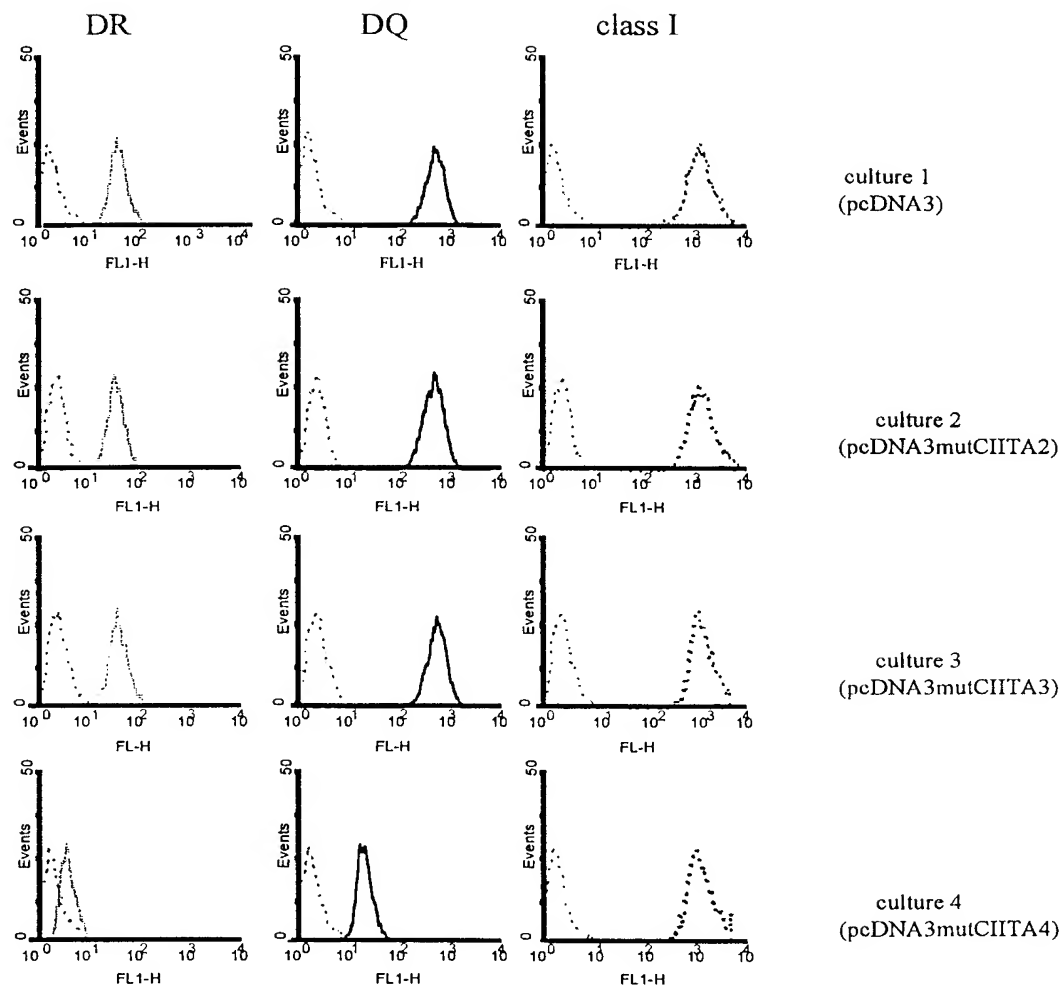


Figure 11

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A



B

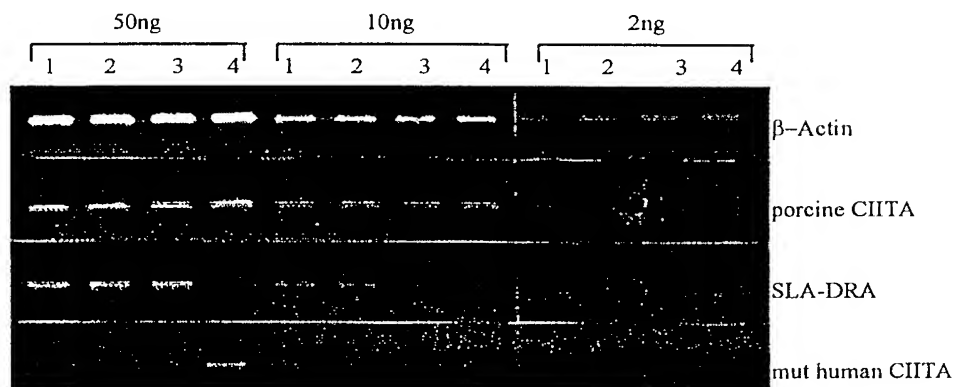


Figure 12

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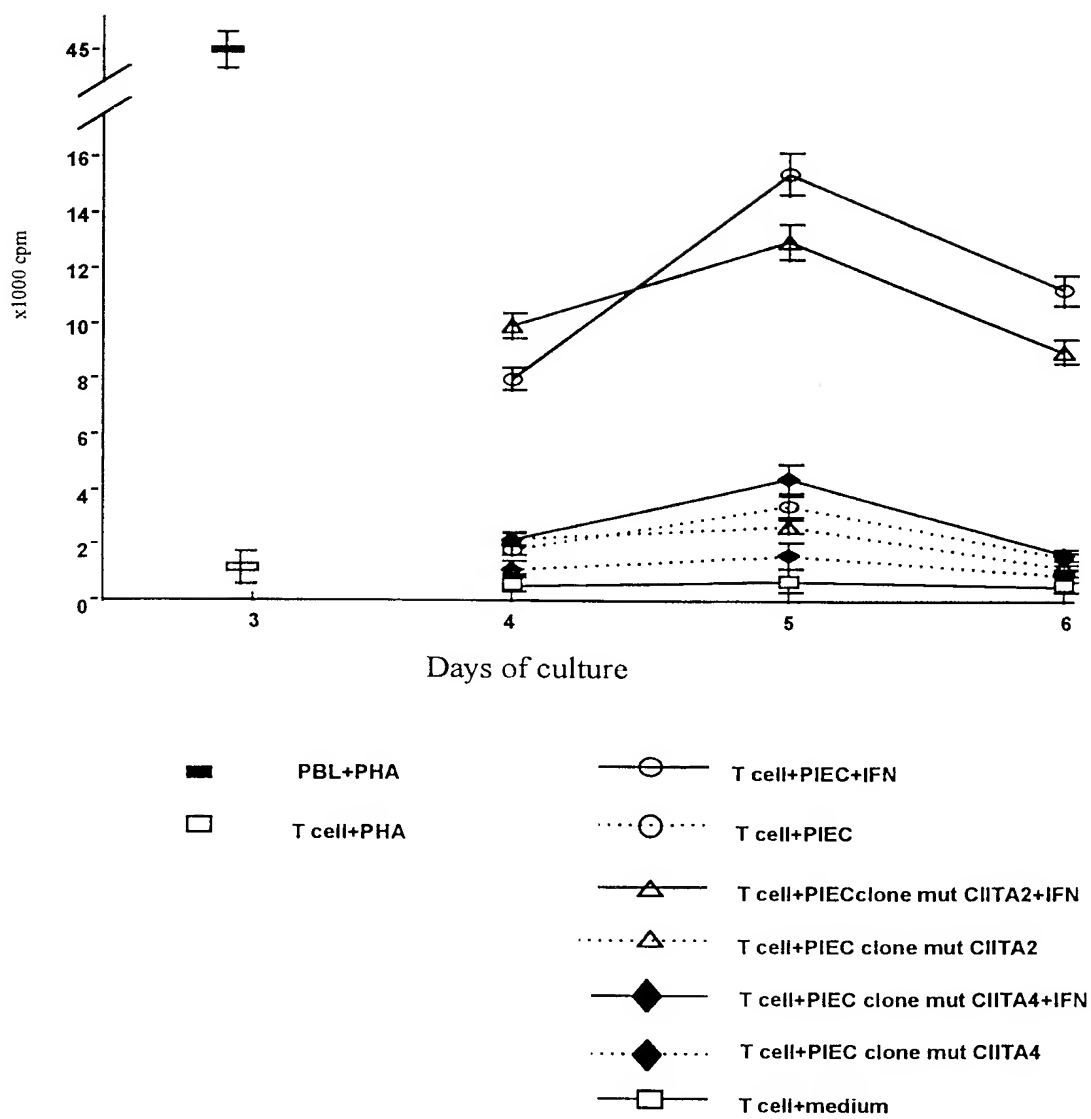


Figure 13

Amino acid position in C11TA

1	2	3	4	5	6	"150"	151	152	153		
(AATT) CTACACA	ATG	CGT	TGC	CTG	GCT	CCA	ATC	ATT	CCG	GCA	-----
M	R	C	L	A	P	I	I				

part of endonuclease restriction site

from 5'UT region of C11TA

previously:

GAG CTT

E L

as

Figure 5

Figure 14